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The research of functional gene delivery using the liposome modified with temperature-responsive polymer (温度応答性高分子を修飾したリポソームによる機能的遺伝子デリバリーに関する研究)

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Preface

RNA interference (RNAi) is a biological process in which RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules. Small interfering RNA (siRNA) plays an important role in RNAi pathway. However, siRNA is unstable and its cellular uptake is difficult. Therefore, it is essential to develop an efficient Drug Delivery System (DDS) carrier for siRNA.

The liposome is one of materials for DDS. At DDS, the liposome needs to have long circulation in blood, efficient release, and good cellular uptake. Furthermore, modification of hydrophilic polymer on surface of liposome, like poly ethylene glycol (PEG) can extend blood circulation and avoid the Reticuloendothelial System (RES). PEG conformed aqueous layer on the surface and this aqueous layer performs these effects. But the aqueous layer restrains interaction of liposomes with target cell. Therefore modification PEG would make liposome difficult to be transferred to intercellular.

In this study, I synthesized the temperature-responsive polymer and developed the temperature-responsive liposome as a novel carrier for siRNA delivery, I prepared lipoplexes and assessed cellular uptake of siRNA and gene silencing activity of target genes.

【Index】

1. Introduction

Drug delivery using nanoparticle carrier systems is an attractive strategy for cancer therapy to enhance therapeutic efficacy while reducing side effects [1]. In particular, PEGylated liposomes prepared by the liposomes conjugated with polyethylene glycol (PEG), have been widely used to enhance the accumulation of liposomes in tumor tissues through enhanced permeability and retention (EPR) effects mediated by their long circulation time [2-5]. However, the hydration surface of PEGylated liposomes creates a major drawback by its limited cellular uptake and cellular release from liposomes [6, 7]. Strategies targeted at overcoming the limited drug release drawback include stimuli-responsive liposomes that trigger drug release using external stimuli such as temperature and pH. In particular, temperature is mostly used to trigger the release of temperature-responsive liposomes [8, 9].

Temperature-responsive liposomes are classified into two types: the traditional temperature-responsive liposomes composed of temperature-responsive lipids and temperature-responsive polymers modified on liposomes [10]. The traditional temperature-responsive liposomes show the greatest permeation of the lipid membrane at its gel-to-liquid crystal phase transition temperature. The temperature-responsive liposomes remain intact at 37 °C and exhibit drug release from the liposomes following raising temperature above 37 °C. One of this type temperature-responsive liposomes loaded with doxorubicin, ThermoDox® reached Phase III trials in 2014 [11]. In addition, liposomes modified with temperature-responsive polymers exhibit a lower critical solution temperature (LCST) behavior compared with the temperature-responsive lipids liposomes. The polymers are soluble in an aqueous solution at a low temperature, but are dehydrated and aggregated by heating above the LCST. Liposomes modified with temperature-responsive polymers show an effective release of encapsulated contents above the polymer LCST. Poly(*N*-isopropylacrylamide) (PNIPAAm) displays an LCST behavior at approximately 32 °C [12, 13]. The LCST of the polymer can be controlled by

copolymerization with other monomers [14, 15]. PNIPAAm and its copolymers have been widely used for the modification of liposomes [16, 17].

In most cases, the temperature-responsive liposomes released the entrapped drug rapidly before cellular uptake following the temperature raised. This results leading drug penetration through the cell membrane by simple diffusion [10]. Nanoparticles and liposomes are uptaked into cells by endocytosis. PEGylated liposomes hardly penetrate cells, and exhibit a limited released of their content to the cells, due to low affinity for the cell membrane because of the steric barrier of polyethylene glycol (PEG) [6, 7]. Temperature-responsive polymer-modified liposomes can be activated by raising temperature. They are useful as intracellular carriers for hydrophilic or hydrophobic, high molecular weight, and biodegradable drugs such as peptide and gene that cannot penetrate membranes, and are expected to enhance the cellular uptake and release their content into cell.

However, the surface property of PNIPAAm copolymer modified liposomes for drug release and cellular uptake have not been reported. To increase cellular uptake, PEGylated traditional temperature-responsive liposomes modified with ligands and antibodies [18-20], as well as temperature-responsive polymer-modified liposomes conjugated with antibody and exposed to ultrasound have been reported [21-23]. Moreover, nanoparticles without liposomes including our temperature-responsive poly-*N*-isopropylacrylamide-polylactic acid (PNIPAAm-PLA) nanoparticles [24], temperature-responsive poly(*ε*-caprolactone) micellar nanoparticles [25], poly-*N*-isopropylacrylamide-*co*-*N,N*-dimethylaminoethyl methacrylate (PNIPAAm-*co*-DMAEMA) nanoparticles [26], PNIPAAm-chitosan modified magnetite nanoparticles [27], and poly(*N*-isopropylacrylamide)-*block*-poly((3-acrylamidopropyl)trimethylammonium chloride) (PNIPAAm-PAMPTMA) polymer complex with siRNA [28] were reported to be cellular uptake to cells with raising temperature. In addition, recently, temperature-responsive

biopolymer elastin-like polypeptide modified liposomes were reported to show induced cellular uptake following the dehydration of the molecules on the liposomal surface [29]. The dehydration of the temperature responsive polymer increases its interaction with the cell membrane, PNIPAAm copolymer modified liposomes are expected to contribute to enhancing both of the cellular uptake and drug release to the cells following raising temperature. (**Figure 1. 1**)

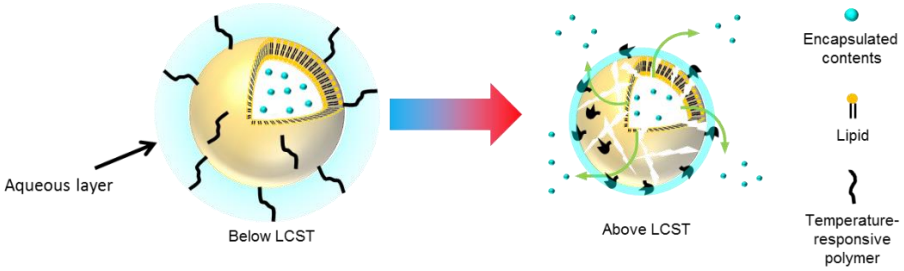

Figure 1. 1. Temperature-induced property change and release of temperature-responsive liposomes across its LCST

Gene-based therapies have been extensively investigated to treat diseases by delivering nucleic acids into cells to induce or silence specific gene expression [30]. Cationic liposomes are the most common lipid-based non-viral nanocarriers for delivery of nucleic acids (small interfering RNA (siRNA), plasmid DNA (pDNA), oligonucleotides, etc.) since the cationic charge can conform complex with the anionic gene fragment and enhance the interaction with cell membrane. In gene delivery, modification of the liposomes with PEG also reduce excessive interaction seems very effective for stabilizing liposomes in the blood stream [2, 31, 32]. Yet, PEGylated liposomes show lower transfection efficiency due to reduced contact between the liposome surface and the cell membrane, and inhibition of endosomal release [6, 7, 33].

To overcome these problems, conditionally cleavable PEG-modified liposomes have been designed that respond to changes of local pH and enzymes [34, 35]. Another approach has

focused on the use of alternative polymers, such as pH-sensitive and temperature-responsive polymers.

PNIPAAm-copolymer-modified liposomes have been mostly reported for triggered release of liposomal drugs [36]. However, in regard to thermally triggered cellular uptake, a few studies including the polymer complexed with nucleic acids have been reported; NIPAAm and 2-(dimethylamino) ethyl methacrylate (DMAEMA)/pDNA complex [37], NIPAAm-conjugated siRNA, quantum dots, cell-penetrating peptide system [38] and PNIPAA-*b*-PAMPTMA (poly((3-acrylamidopropyl)trimethylammonium chloride)) diblock copolymer/siRNA complexes [28]. To the best of our knowledge, no reported studies have developed gene delivery using PNIPAAm copolymer-modified liposomes.

In this study, I synthesized poly-*N*-isopropylacrylamide-*co*-*N,N*'-dimethylaminopropylacrylamide (P(NIPAAm-*co*-DMAPAAm) -1,2-dioleoyl-sn-glycero-3-phospho-ethanolamine (*L*-*α*-phosphatidylethanolamine dioleoyl; DOPE), and compared the physicochemical properties, drug release, and cellular uptake efficiency of the DOPE conjugated P(NIPAAm-*co*-DMAPAAm) modified *N*-[1-(2, 3-dioleoyloxy) propyl]-*N,N,N*-trimethyl-ammonium methylsulfate (DOTAP)/DOPE liposomes as temperature-responsive liposomes with those of hydrated PEGylated liposomes, to develop intracellular drug carriers. The DOTAP could induce the liposome conforming lipoplex with siRNA for its cationic charge, and the DOPE can increase the interaction with cells by its membrane fluidity effect. Here, I showed that the tunable surface properties of the temperature-responsive polymer-modified liposomes enabled their heat-activated dehydration, which induced faster cellular uptake and release. Moreover, to develop temperature-responsive liposomes as a novel carrier for siRNA transfection, I prepared siRNA lipoplexes and assessed cellular uptake of siRNA and its gene silencing activity of target genes, compared with those of a conventional transfection reagent, Lipofectamine RNAiMAX, and non-modified or PEGylated liposomes below and near the

LCST. The cellular uptake mechanism of siRNA lipoplexes was examined using endocytosis inhibitors.

2. Synthesis of the lipid conjugated temperature-responsive polymer: P(NIPAAm-*co*-DMAPAAm)-DOPE

2.1. Materials

N-isopropylacrylamide (NIPAAm) • • • • • • • • • • • • • KJ Chemicals Co. (Tokyo, Japan)

N,N'-dimethylaminopropylacrylamide (DMAPAAm) • • KJ Chemicals Co. (Tokyo, Japan)

2,2'-azobisisobutyronitrile (AIBN) • Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

3-mercaptopropionic acid (MPA) • • Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

N-hydroxysuccinimide (NHS) • • • • • • • • • • • • • • Kanto Chemical Co. (Tokyo, Japan)

N,N'-dicyclohexylcarbodiimide (DCC) • • • • • • • • • • Kanto Chemical Co. (Tokyo, Japan)

Dimethyl formaldehyde (DMF) • • • Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

Dioxane • • • • • • • • • • • • • • • Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

L-*α*-phosphatidylethanolamine, dioleoyl (DOPE) •

• Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

DMF containing 10 mM LiCl • TOSOH (Tokyo, Japan)

2.2. Methods

2.2.1. Preparation and Characterization of temperature-responsive polymer

We designed a temperature-responsive polymer based on previous reports of PNIPAAm copolymer [14, 15, 39]. The synthesize scheme of the synthesis of P(NIPAAm-*co*-DMAPAAm)-DOPE is shown in **Figure 2. 1**. NIPAAm and DMAPAAm at a fixed molar ratio of 95/5 (total 88 mmol), MPA (2.4 mmol), and AIBN (0.35 mmol) were mixed with dimethylformamide (20 mL) and heated at 70 °C for 5 h under nitrogen gas (N_2). After copolymerization, the copolymer was recovered using diethyl ether. To conjugate this copolymer with DOPE, I first replaced the terminal carboxyl group with an active ester.

The copolymer (0.2 mmol), NHS (0.5 mmol), and DCC (0.5 mmol) were dissolved in dichloromethane (10 mL). The copolymer and DOPE were mixed with dioxane at a fixed molar ratio of 1/1, and reacted at 25 °C for 24 h.

The molecular weight of P(NIPAAm-*co*-DMAPAAm) was determined by gel permeation chromatography (GPC) analysis (GPC-8020 system; column, TSK-GEL; mobile phase, DMF containing 10 mM LiCl), calibrated with polyethylene oxide standards.

The chemical component was determined by ^{1}H NMR, by estimation from the integrated proton signals derived from NIPAAm methane (1H, 4.0 ppm), DMAPAAm methyl (6H, 2.9 ppm), and DOPE methane (1H, 5.3 ppm) in $CDCl_3$.

The copolymer LCST in water was evaluated using transmittance measurement and differential scanning calorimetry (DSC). The transmittance of the copolymer aqueous solution (5 mg/mL) at wave length 500 nm was monitored using a spectrophotometer (V-630, JASCO Corp., Tokyo, Japan) with a water-jacketed cell holder coupled with a circulating bath. The temperature was raised at a rate of 0.1 °C/min. The LCST was considered as the temperature at the 50% point in the resulting transmittance vs. temperature curve. The transition of the copolymer aqueous solution (100 mg/mL) was monitored using the DSC-60Plus (Shimadzu Corp., Kyoto, Japan). The aluminum pan was sealed using a tablet press machine, and the heating scan was carried out from 20 to 60 °C at a scanning rate of 1 °C/min under dry N_2. The LCST was considered as the temperature at the peak of the DSC.

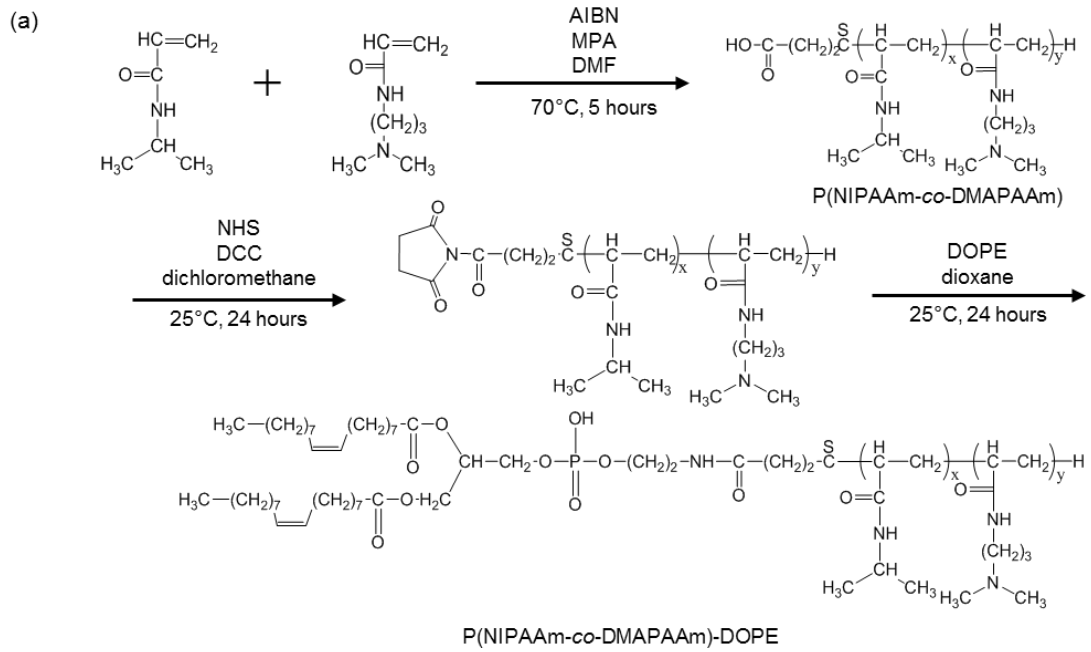

Figure2. 1. The synthesize scheme of temperature-response polymer, P(NIPAAm-*co*-DMAPAAm) conjugation to DOPE

2.3. Results and discussion

2.3.1. Characterization of temperature-responsive polymer.

The NIPAAm and DMAPAAm in the P(NIPAAm-*co*-DMAPAAm) acted as the temperature-responsive and hydrophilic moieties. In aqueous solution, PNIPAAm exhibits an expanded coil conformation below the LCST because it is strongly hydrated, but it changes to a globular form above the LCST due to its sudden dehydration [40]. It was reported that the coil-globule transition of PNIPAAm could be detected using DSC.

The molecular-weight of the P(NIPAAm-*co*-DMAPAAm) was determined by GPC, and the molecular-weight was 5500.

The chemical component was determined by ^{1}H NMR. The ^{1}H NMR spectrum was shown in **Figure 2. 2**.

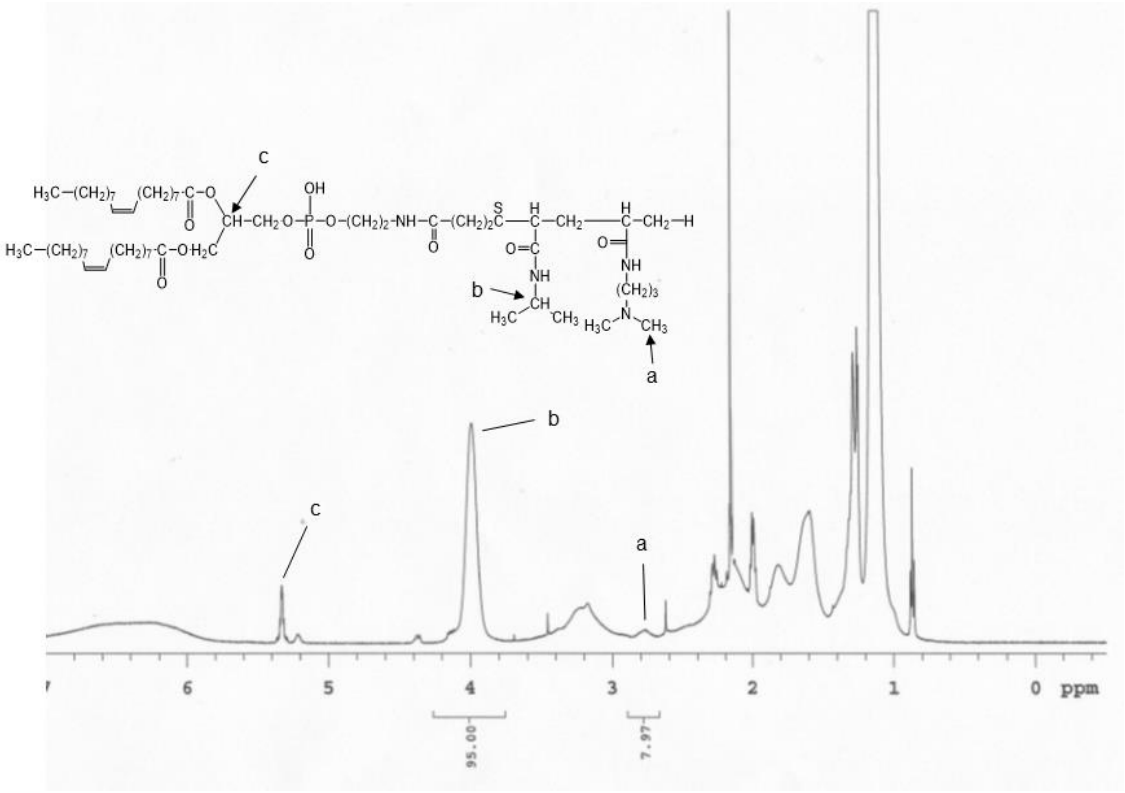

Figure2. 2. P(NIPAAm-*co*-DMAPAAm)-DOPE was determined by ^{1}H NMR, by estimation from the integrated proton signals derived from NIPAAm methane (1H, 4.0 ppm), DMAPAAm methyl (6H, 2.9 ppm), and DOPE methane (1H, 5.3 ppm) in $CDCl_3$.

2.3.2. The LCST of temperature-responsive polymer.

The temperature-responsive transition of the P(NIPAAm-*co*-DMAPAAm) suspension in water was examined using DSC and transmittance measurement. The DSC curve showed a large and sharp endotherm centered at 40.2 °C (**Figure 2. 3a**) while the transmittance curve with a 50% transmittance was observed at 40.5 °C (**Figure 2. 3b**). These results indicate that the copolymer chains have a sharply transition point from a hydrophilic to a hydrophobic state at approximately 40 °C. Therefore, the LCST of P(NIPAAm-*co*-DMAPAAm) was determined to be 40 °C, which is above the normal body temperature.

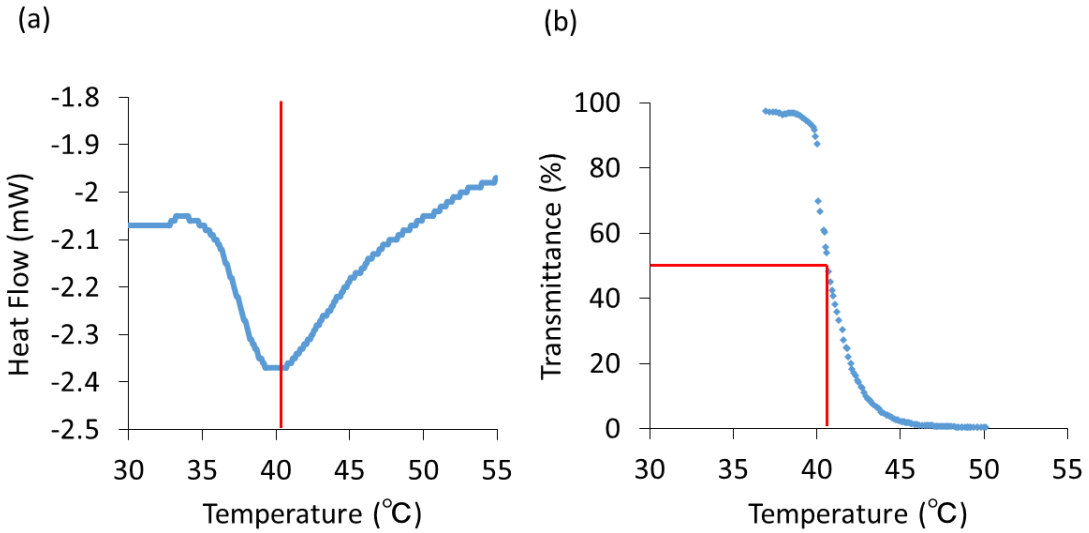

Figure2. 3. LCST of P(NIPAAm-co-DMAPAAm) in water determined using (a) differential scanning colorimetry (DSC) and (b) transmittance measurement.

3. Characterization and cellular uptake about temperature-responsive liposomes

3.1. Materials

N-[1-(2,3-Dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl-sulfate (DOTAP)・・・・・・・・・・・・・・・・・・・・・・・・・Avanti Polar Lipids Inc. (Alabaster, AL, USA)

L-*α*-phosphatidylethanolamine, dioleoyl (DOPE)・・・・・・・・・・・・・・・・・・・・・・・・・・・・・・・・・Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

N-[methoxy (polyethylene glycol)2000]-distearoyl phosphatidylethanolamine (PEG-DSPE)・・・・・・・・・・・・・・・・・Avanti Polar Lipids Inc. (Alabaster, AL, USA)

Rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (rhodamine-DHPE)・・Life Technologies Co. (Carlsbad, CA, USA)

5(6)-carboxyfluorescein (CF)・・・・・・・・・・ Sigma-Aldrich Corp. (St. Louis, MO, USA)

Minimum essential medium (MEM)・・・・Thermo Fisher Scientific (Waltham, CA, USA)

Bovine serum (FBS)・・・・・・・・・・・・・・・・・・・・・・・・・Biosera (Sussex, UK)

Penicillin-Streptomycin Glutamine・・・・・Thermo Fisher Scientific (Waltham, CA, USA)

MEM Non-Essential Amino Acids (NEAA)・・・・・・・・・・Thermo Fisher Scientific (Waltham, CA, USA)

Dulbecco's phosphate-buffered salines (PBS)・・・・・・・・・・Thermo Fisher Scientific (Waltham, CA, USA)

0.05 w/v% Trypsin-0.53 mmol/L EDTA・4Na・・・・Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

Water-soluble tetrazolium (WST)-8・・・・・・・・・・・・・・(Dojindo, Kumamoto, Japan)

Sucrose・・・・・・・・・・・・・・・Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

Cytochalasin D・・・・・・・・・・・Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

Nocodazole・・・・・・・・・・・・・Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

Filipin III・・・・・・・・・・・・・・・・・・・・Cayman Chemical (Ann Arbor, MI, USA)

3.2. Methods

3.2.1. Preparation of temperature-responsive liposomes.

The liposomes were prepared with DOTAP, DOPE, and P(NIPAAm-*co*-DMAPAAm)-DOPE or PEG-DSPE each combined at a fixed molar ratio of 30/65/5 mixed in chloroform solution, when necessary, rhodamineB-DHPE (0.01 mol%) added to chloroform solution, the solution in the flask was subsequently evaporated. The non-modified liposomes were prepared with DOTAP and DOPE (1:1 molar ratio). The thin lipid and polymer membrane were dispersed in 1 mL of H_2O, PBS (pH 7.4) or 2 mM CF in PBS to prepare the empty or CF-entrapped liposomes, respectively. To prepare the particle size of liposomes more smaller, the sonication was done using a bath sonicator. The liposomes were extruded through a polycarbonate membrane with 100-nm pore diameter. The free lipid, polymer, and CF were removed using gel permeation chromatography on a SephadexG-25M column using H_2O or PBS. The liposomes were kept at 4 °C until they were analyzed.

3.2.2. The particle size about temperature-responsive liposomes.

The colloidal stability of the liposomes was evaluated by measuring the particle size of the suspensions. The particle size of the temperature-responsive, PEGylated, and non-modified liposomes at room temperature was measured the mean particle size at 25 °C as the colloidal stability using dynamic light scattering (DLS) with a Zetasizer Nano-ZS (Malvern Instruments Ltd., Worcestershire, UK), respectively. The particle size change dependent on temperature change was measured with the temperature-induced change

from 30 °C to 50 °C.

3.2.3. Determination of CF release from temperature-responsive liposomes.

CF is a hydrophilic small molecular fluorescent substance, and easy to observe for the release of liposome encapsulated contents. The CF entrapped liposome suspension was added to PBS in a quartz cell (final CF concentration was 1 μM) at a given temperature, and the fluorescence intensity of the solution was monitored using a spectrometer. The excitation and emission wavelengths were 492 and 518 nm, respectively. Because the liposomes rapidly released the contents at temperatures above LCST, it was difficult to estimate the initial intensity of the CF-entrapped liposomes suspension. We considered the fluorescence intensity at 20 °C as the standard release for the CF release measurement as previously described [9]. Furthermore, the percentage release of CF was defined as

$$\text{Release (\%)} = \{(F \times F_0'' / F'') - F_0'\} / (F_0'' - F_0') \times 100 \quad \text{Eq. 2}$$

Where, F, F_0', and F_0'' are the intermediary, initial, and final fluorescence intensities, respectively while F'' is the final fluorescence intensity at each temperature. F'' and F_0'' were obtained as the fluorescence intensity of the liposomes suspension after the rupturing of liposomes was induced by the addition of Triton X-100. The change in CF release from the liposomes was measured from 30 °C to 45 °C.

3.2.4. Measurements of zeta potential and FALT of temperature-responsive liposomes.

The zeta potentials of the liposomes were measured using dynamic light scattering (DLS) with the electrophoretic light scattering ELSZK-2 KOP apparatus (Otsuka Electronics Co., Ltd., Osaka, Japan). To calculate the fixed aqueous layer thickness (FALT) of each liposome, the liposomes suspension was dispersed in 9% sucrose solution.

We calculated the FALT using the Gouy–Chapman theory [41, 42]. According to this theory, the zeta potential $\psi(L)$, calculated as the electrostatic potentials at the position of the slipping plane L (nm) was expressed as:

$$\ln\psi(L) = \ln A - \kappa L \qquad \text{Eq. 1}$$

Where A is a constant; κ is the Debye–Hückel parameter, which = $\sqrt{C}0.3$ for univalent salts; and C is the molality of electrolytes. When zeta potentials are measured from the changing concentration of sodium chloride (NaCl) and plotted against κ, the slope, L gives the position of the slipping plane or FALT in nanometers. Based on this theory, the FALT of each liposome was estimated from 30 °C to 45 °C.

3.2.5. Cell culture.

RAW264.7 macrophage and human epithelioid cervix carcinoma HeLa cells (RIKEN BRC Cell Bank, Ibaraki, Japan) were cultured as subconfluent monolayers in a 75-cm^2 culture flask with a vent cap using minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin, and 50 μg/mL streptomycin at 37 °C in a humidified incubator exposed to 5% CO_2. Subconfluent RAW264.7 cells were dissociated using a cell scraper (30-cm, TPP, Switzerland) and cultured in a flask for 2-3 days. The subconfluent HeLa cells were dissociated using 0.05 w/v% trypsin 0.53 mmol/L ethylenediaminetetraacetic acid (EDTA)·4Na solution and cultured in a flask for 3-4 days.

3.2.6. Determination of cellular uptake of temperature-responsive liposomes using flow cytometry.

To detect the cellular association of the liposomes, the RAW264.7 cells were seeded in a 60-mm dish at a density of 5.0×10^5 cells per dish, in 5 mL of the medium. After an

overnight incubation, the cells were further incubated for 30 min with rhodamine-labeled PEGylated liposomes and rhodamine-labeled temperature-responsive liposomes at 30 °C or 40 °C. After incubation, the RAW264.7 cells were washed twice with PBS, harvested with trypsin/EDTA, resuspended in PBS, and the cell-associated fluorescence was subsequently detected using a flow cytometer (BD LSRII, BD Biosciences, San Jose, CA, USA) equipped with a filter appropriate for the detection of 575/26 bandpass filter.

3.2.7. Evaluation of cellular uptake of CF encapsulated temperature-responsive liposomes using fluorescence microscopy.

HeLa and RAW264.7 cells (1×10^5 and 2×10^5 cells/dish, respectively) were grown in 2 mL of the medium with 10% FBS. After an overnight incubation, 50 μL rhodamine-labeled liposomes entrapped with CF was added and incubated at 30 °C, 37 °C, or 40 °C for 30 min. Then, the cells were rinsed thrice with PBS, fixed with 4% paraformaldehyde at 4 °C for 20 min, rinsed thrice with PBS, and observed using a fluorescence microscope (BZ-9000, Keyence Corp., Osaka, Japan).

3.2.8. Cellular uptake of temperature-responsive liposomes after treatment with inhibitors of endocytosis.

HeLa cells (5.0×10^4 cells/well) were grown in 2 mL of the medium by plating them on 6-well culture plates 24 h before each experiment. For the inhibitors pretreatment, I incubated the cells at 4 °C or with medium containing dissolved cytochalasin D (5 μg/mL), nocodazole (3 μg/mL), sucrose (69 mg/mL), and filipin III (2 μg/mL) at 37 °C for 1 h. Subsequently, 30 μL rhodamine-labeled liposomes were added to the medium, and further incubated at 40 °C for 30 min with 10% FBS. After incubation, the cells were washed twice with PBS, harvested with trypsin/EDTA, resuspended in PBS, and the cell-

associated fluorescence of rhodamine was subsequently detected using a flow cytometer equipped with a filter appropriate for the detection of 575/26 bandpass filter.

3.2.9. Cell viability assay.

HeLa cells (5.0×10^3 cells/well) were grown in 2 mL of the medium by plating them on a 96-well culture plate. Each liposome was diluted in 100 μL medium supplemented with 10% FBS, and the mixture was applied to the cells. After a 24 h incubation at 37 °C and 40 °C, 10 μL WST-8 was applied to each well and incubated at 37 °C for 1 h. The absorbance at 450 nm was measured using a microplate reader (TECAN infinite M-1000, Tecan Japan Co. Kanagawa, Japan).

3.2.10. Statistical analysis.

The Student's non-paired *t*-test was used to compare the normally distributed values between the groups. A $p < 0.02$ was considered statistically significant.

3.3. Results and discussion

3.3.1. The particle size about temperature-responsive liposomes.

The colloidal stability of the liposomes with temperature change was evaluated by measuring the particle size of the suspensions (**Figure 3. 1**). The particle size of the non-modified, PEGylated, and temperature-responsive liposomes at 25 °C was 125 ± 0.3 nm, 145 ± 0.7 nm, and 118.2 ± 0.2 nm at (mean ± standard deviation, SD), respectively (**Table 3. 1**). When the temperature was increased, the particle size of the temperature-responsive liposomes did not increase until 39 °C, suggesting that the liposomes were

stable at physiological temperatures, below the copolymer LCST (40 °C). However, above the LCST, the particle size of the liposomes increased markedly. This result suggested that the liposomes had a hydrophobic surface and lost their colloidal stability above the LCST, and formed aggregate. On the other hand, the PEGylated and non-modified liposomes were stable throughout the temperature region.

The destabilization by aggregation of the temperature-responsive liposomes were induced by the temperature-dependent characteristic change of the copolymer. The DOTAP/DOPE (3:7) liposomes were unstable because DOPE induced the fluidity of lipid membrane. However, below the LCST, the temperature-responsive had the hydrated copolymer chains liposome similar to the PEGylate liposomes, may sufficiently cover the liposomes surface and, thereby, increase the stability of the unstable DOTAP/DOPE (3:7) liposomes. However, above the LCST, the dehydrated copolymer chains may be distributed partly at the liposomal surface, and therefore, may not cover the liposomes surface sufficiently, which would greatly decrease the stability of the liposomes. So the copolymer LCST appeared change of the surface property of temperature-responsive liposomes with the copolymer change, causing liposomes to aggregate.

Table 3. 1. The mean particle size and polydispersity index (PDI) about non-modified, PEGylated, and temperature-responsive liposomes.

	Mean particle size	**PDI**
Non-modified liposome	118 ± 0.2 nm	0.17
PEGylated liposome	145 ± 0.7 nm	0.18
P(NIPAAm-co-DMAPAAm)-modified liposome	125 ± 0.3 nm	0.25

*PDI: Width of particle size distributions.

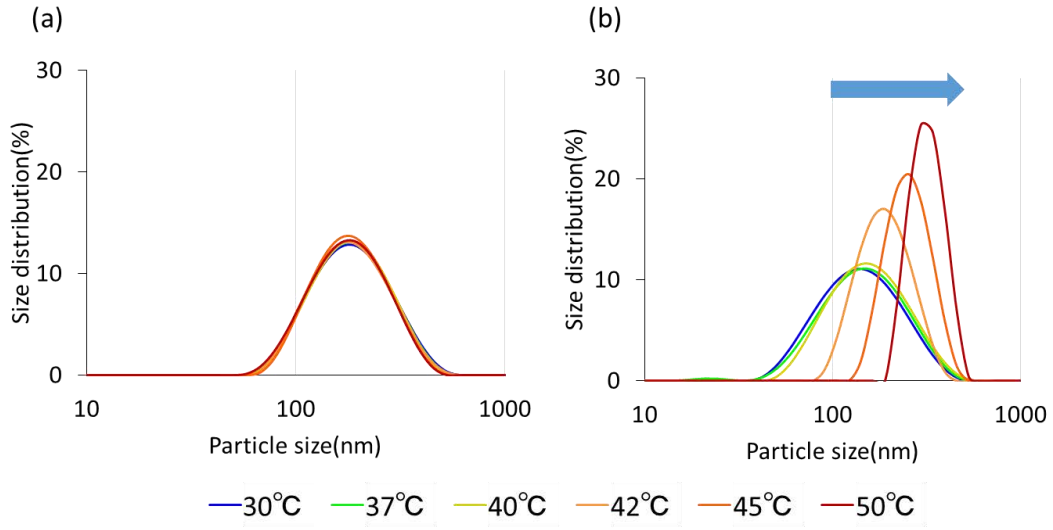

Figure 3. 1. Change in particle size of liposomes suspension in phosphate-buffered saline (PBS) with increasing temperature PEGylated (a), and poly-*N*-Isopropylacryamide-*co*-*N,N′*-dimethylaminopropylacrylamide [P(NIPAAm-*co*-DMAPAAm)] modified (b) liposomes.

3.3.2. Determination of CF release from temperature-responsive liposomes.

The CF release from CF-entrapped temperature-responsive liposomes was measured after incubation at different temperatures compared with other liposomes (**Figure 3. 2**). The PEGylated (**Figure 3. 2a**) and non-modified (**Figure 3. 2c**) liposomes released CF content tightly throughout the tested temperature region. The temperature-responsive liposomes also released the CF content tightly below 37 °C and released less than 15% in 30 min at 37 °C (**Figure 3. 2b**). However, the CF release increased markedly at temperatures > 39 °C, near the LCST, and at 42 °C, which was above the LCST. They released approximately 80% of the CF in 30 min (**Figure 3. 2b**).

The increasing of content release by the temperature-responsive liposomes were induced by the temperature-dependent characteristic change of the copolymer. Below the copolymer LCST, similar to the PEGylate liposomes, the liposomes surface was be covered with the hydrated copolymer chains that increasing the stability liposomes.

However, above the LCST, the liposome may not be covered with the dehydrated copolymer chains, which would greatly decrease the stability of the membrane of liposomes. So the copolymer LCST related the temperature of the liposomes release their content.

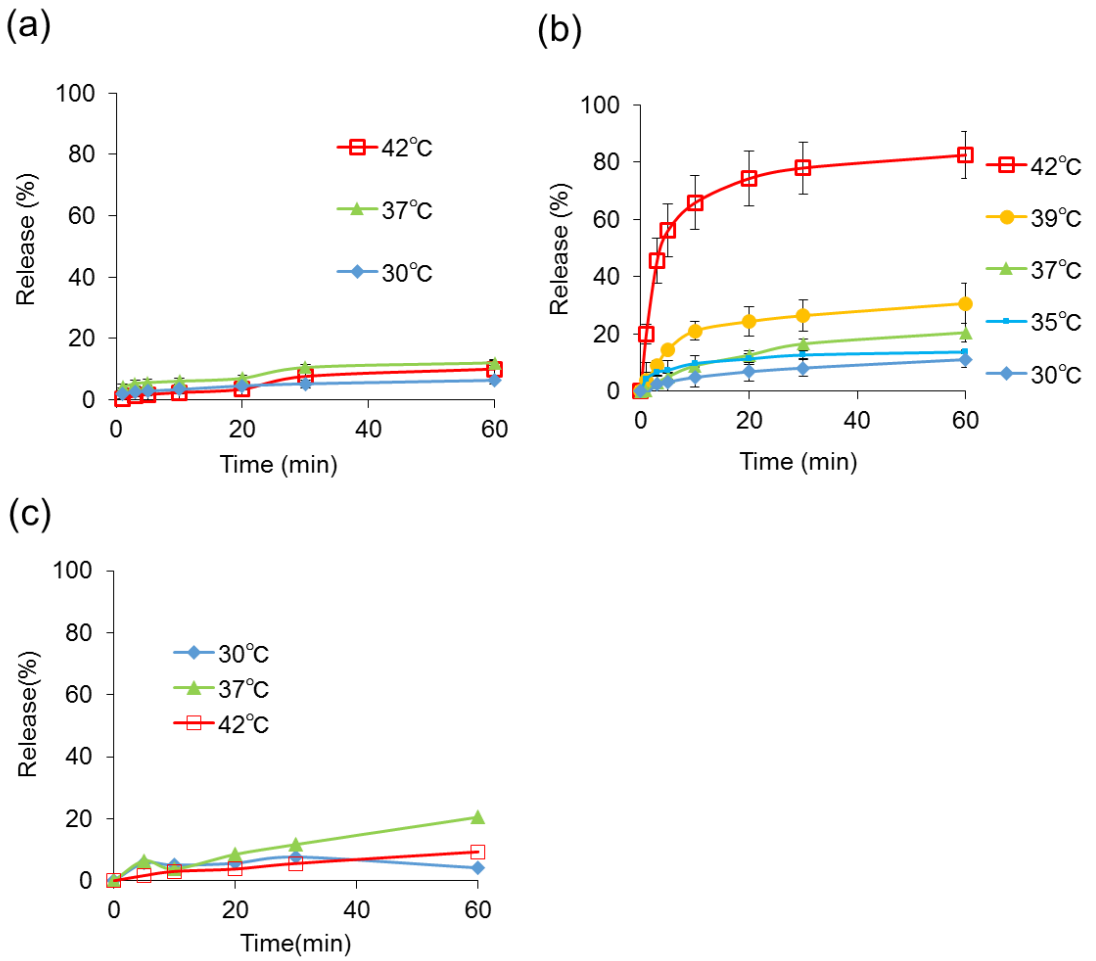

Figure 3. 2. Carboxyfluorescein (CF) release (%) in suspension of (a) PEGylated liposomes, (b) P(NIPAAm-*co*-DMAPAAm)-modified liposomes, and (c) non-modified liposomes in PBS from 0 to 60 min at each temperature; mean± standard deviation (SD, n = 3).

3.3.3. The zeta potential and change in FALT of temperature-responsive liposomes

To investigate the surface properties of the temperature-responsive liposomes, I measured the zeta potential of temperature-responsive liposomes at different temperatures (**Table 3. 2**). All the liposomes in this study exhibited a positive zeta potential, resulting from the cationic lipid, DOTAP. In the result, the temperature-responsive liposomes showed a lower zeta potential than the non-modified and PEGylated liposomes. With increasing temperature, the zeta potential of the temperature-responsive liposomes showed an increasing while the non-modified and PEGylated liposomes did not over the tested temperature region (**Table 3. 2**).

The zeta potential values of temperature-responsive liposome increased when the NaCl concentration was increased **(Table 3. 2)**. The calculated FALT (Fixed aqueous layer thickness) values are shown in **Figure 3. 3**. The value of the non-modified liposomes was low (0.17 nm) while that of the PEGylated liposomes was high (4.4 nm). Then the value did not dependent temperature change at this range. The FALT of the PEGylated liposomes was in agreement with the 3.5 nm value described previously [43]. The temperature-responsive liposomes also showed a FALT of approximately 3.7 nm at 30 °C, which was comparable to that of the PEGylated liposomes. But at 45 °C, the temperature above LCST, the FALT decreased to 0.3 nm, indicating a dehydration of the surface of liposomes. Based on the results indicating that the surface of the liposomes was dehydration above LCST, I propose that the temperature-responsive liposomes have the potential to have colloidal stability, enhance cellular uptake and drug release by temperature control.

Table 3. 2. Zeta (ζ) potential of polymer- and non-modified liposomes in water.

ζ potential (mV)	30 °C	37 °C	45 °C
Non-modified liposome	61.7 ± 0.3	64.4 ± 0.4	62.4 ± 2.9
PEGylated liposome	53.5 ± 4.7	54.1 ± 4.3	61.0 ± 2.2
P(NIPAAm-co-DMAPAAm)-modified liposome	28.2 ± 0.4	38.7 ± 0.3	47.9 ± 1.3

Mean ± SD, n = 3

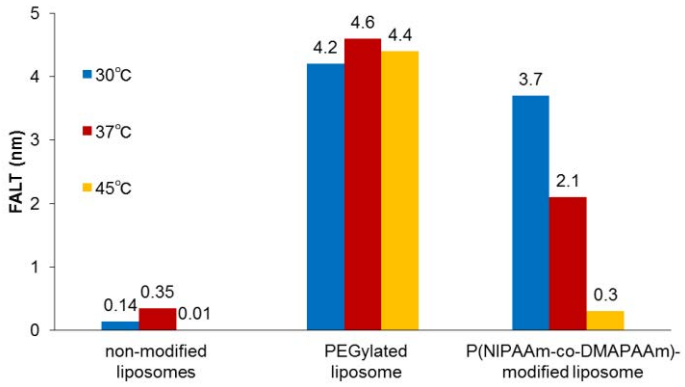

Figure 3. 3. The change of fixed aqueous layer thickness (FALT) about PEGylated, P(NIPAAm-*co*-DMAPAAm)-modified, and liposomes.

3.3.4. The cellular uptake of temperature-responsive liposomes observed by flow cytometer

The flow cytometry revealed that incubation of RAW264.7 cells with rhodamine-labeled PEGylated and P(NIPAAm-co-DMAPAAm) modified liposomes, and both of the liposomes increased the fluorescence intensity at 40 °C compared with 30 °C (**Figure 3. 4a** and **b**). I also calculated the ratio of the fluorescence intensity of rhodamine-labeled liposomes at 30 °C and compared it to that at 40 °C, above body temperature for both liposomes. The increased ratio of fluorescence intensity, i.e., the association ratio of the temperature-responsive and PEGylated liposomes was 2.22 and 1.48, respectively (**Figure 3. 4c**). The cellular association of the rhodamine-labeled temperature-responsive liposomes was more highly increased significantly in a temperature-dependent manner than that of the PEGylated liposomes was ($p < 0.02$). This finding indicates that the

temperature-responsive liposomes showed an efficient cellular association at 40 °C, above the copolymer LCST than they did at 30 °C, compared with the PEGylated liposomes. It is suggested that the dehydrated temperature-responsive liposomes may exhibit enhance the interaction with cell membrane dependent the temperature increasing, and increased the cellular uptake above the LCST. However, the temperature-independent hydrated PEGylated liposomes had a poor interaction with cell membrane compared with temperature-responsive liposome when the temperature increased.

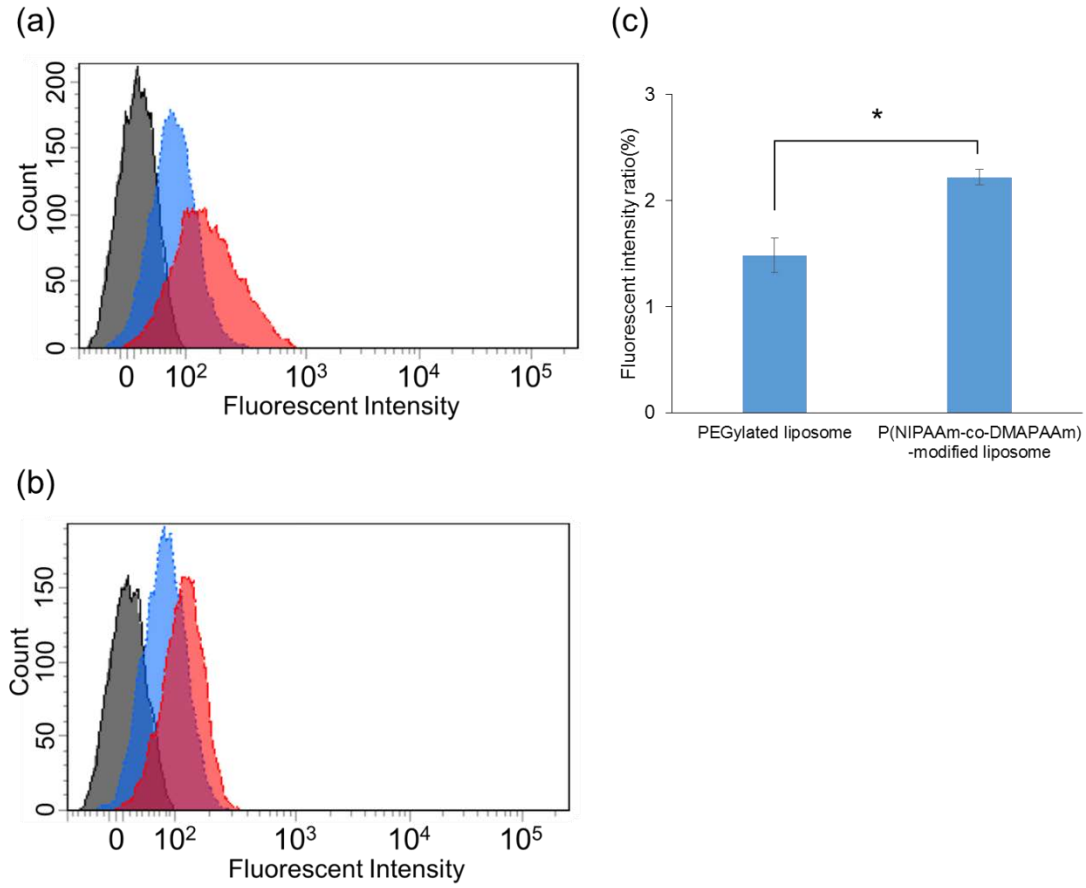

Figure 3. 4. Cellular association of rhodamine-labeled (a) P(NIPAAm-*co*-DMAPAAm)-modified and (b) PEGylated liposomes incubated with RAW264.7 cells for 30 min at 30 °C (blue) and 40 °C (red). Effect of increasing incubation temperature from 30 °C to 40 °C on cellular association of rhodamine-labeled PEGylated and P(NIPAAm-*co*-DMAPAAm)-modified liposomes (c). Rhodamine fluorescence intensity ratio indicated following incubation of each liposome at 40 °C/30 °C Mean ± S.D., n = 3; $p < 0.02$.

3.3.5. The cellular uptake of temperature-responsive liposomes observed by fluorescent microscopy

I further confirmed the cellular uptake of the rhodamine labeled CF entrapped liposomes in RAW264.7 and HeLa cell lines incubated for 30 min. The cellular uptake of free CF or the mixture of free CF and empty temperature-response liposomes did not induce a green fluorescence by the CF in RAW264.7 cells. This indicates that the CF could not penetrate the cells following incubation for 30 min (data not shown).

The PEGylated liposomes incubated with RAW264.7 cells at 37 °C (**Figure 3. 5a**) and displayed a punctate fluorescence induced by the CF (green) and rhodamine (red) and their colocalization (yellow) on the cell surface, which was greater with the HeLa than it was with the RAW264.7 cells (**Figure 3. 5b**). The results suggested that both of the PEGylated liposome and temperature-responsive liposomes incubated at 30 °C did not release the contents into cells and associated with the cell surface and strong localization of rhodamine on the cell surface by electrostatic interaction (**Figure 3. 5c** and **e**). However, the incubation at 40 °C, the CF fluorescence spread dramatically in the cytosol (**Figure 3. 5d** and **f**), indicating that CF was released in cells from the temperature-responsive liposomes following temperature raising. I attributed this increase in fluorescence to the leakage of CF from the liposomes and their dilution in the cell interior as described previously [20].

The observation of CF release from the temperature-responsive liposomes in the cells indicates that the cellular uptake was enhanced by the disappear of aqueous layer and dehydration of the surface induced by the transition of P(NIPAAm-*co*-DMAPAAm) with temperature raising, compared with the PEGylated liposomes taken up into the cells did not release CF. Furthermore, the higher association of liposomes with HeLa cells than RAW264.7 cells may be due to the strong interaction between the cationic liposomes and

the negatively charged cell membrane.

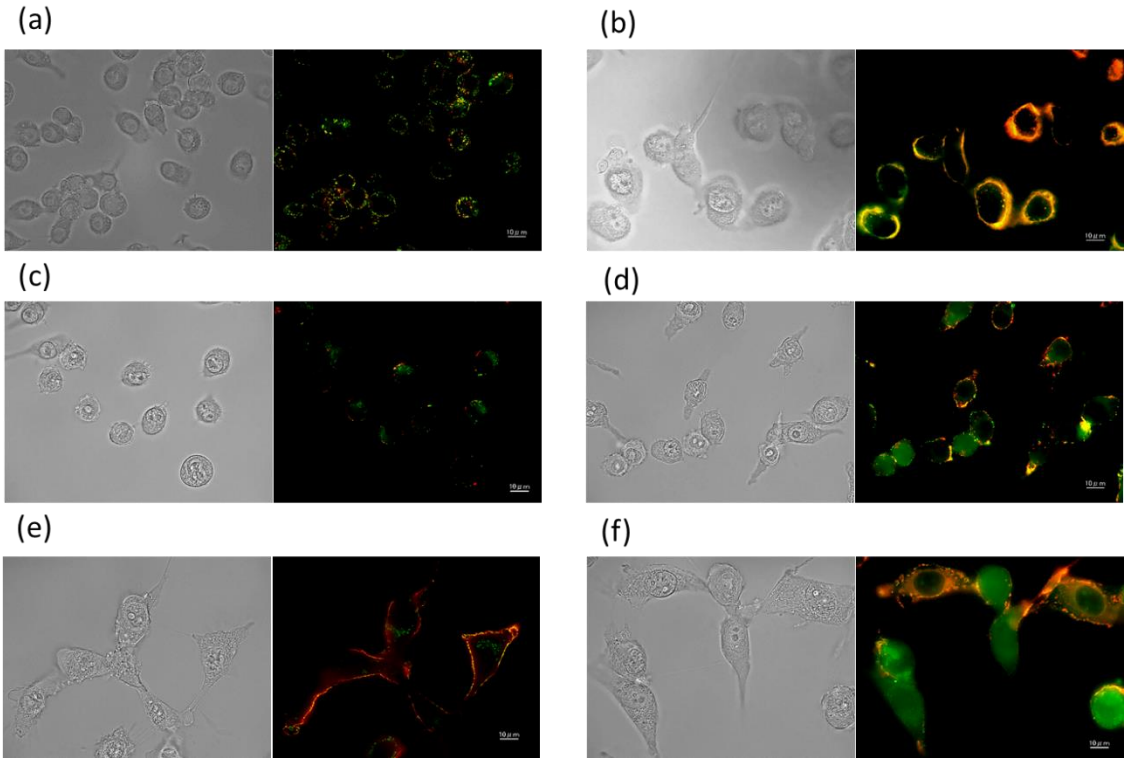

Figure 3. 5. Fluorescence micrograph of carboxyfluorescein (CF)-entrapped liposomes labeled with rhodamine in (a, c, and d) RAW264.7 and (b, e, and f) HeLa cells. PEGylated liposomes incubated at (a and b) 37 °C and P(NIPAAm-*co*-DMAPAAm)-modified liposomes incubated at (c and e) 30 °C and (d and f) 40 °C for 30 min. CF is green, and rhodamine is red. Scale bar, 10 μm.

3.3.6. The cell viability of temperature-responsive liposomes

Cell cytotoxicity by cellular uptake each liposome with HeLa cells was examined. Non-modified liposomes showed significantly low viability at 37 and 40 °C, 35% and 22%, respectively. However, the temperature-responsive liposomes showed high viability (108% and 88%), similar to PEGylated lipoplexes (115% and 91%) at 37 and 40 °C, respectively (**Figure 3. 6**).

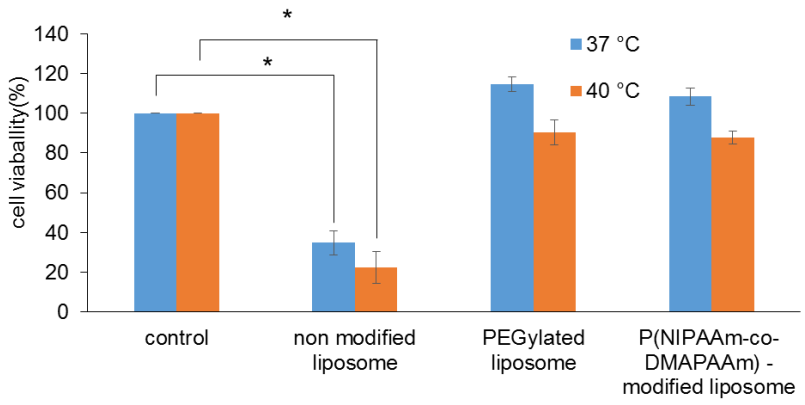

Figure 3. 6. Comparison of viability of cells incubated with non-modified, PEGylated, and P(NIPAAm-*co*-DMAPAAm) modified liposome at 37 °C and 40 °C. Mean ± S.D., n = 3; $p < 0.01$ vs control.

3.3.7. Cell uptake mechanism.

I also investigated the uptake mechanism of the temperature-responsive liposomes. I used cells that were incubated with the liposomes in the absence of inhibitors as the control. The endocytic inhibitors and concentrations were determined based on previously reported studies in the literature [44]. The inhibitor concentrations that showed cell viability ≥ 80% were selected for this study. Cytochalasin D, nocodazole, sucrose, and filipin III are inhibitors of actin-based transport, microtubular transport, clathrin-mediated endocytosis, and caveolin-mediated endocytosis pathways, respectively. The cell internalization of the liposomes was mainly an energy-dependent process because approximately 65% of the cellular uptake was significantly blocked at 4 °C (**Figure 3. 7**). The decrease in the measured rhodamine fluorescence compared to the control, indicated the involvement of specific endocytotic mechanism mediated by the inhibitors (**Figure 3. 7**). A significant inhibition of the uptake with the temperature-responsive liposomes was observed for sucrose (28.5%), which is a clathrin-mediated endocytosis pathway inhibitor ($p < 0.05$) and nocodazole (35.6%), which causes microtubule disruption ($p < 0.01$). The

uptake inhibition by nocodazole suggests that the microtubules participate in the uptake of liposomes that are known to participate in microtubule-dependent transport [45].

These data showed that the cellular uptake of the temperature-responsive liposomes into HeLa cells was contributed to microtubule-dependent transport and clathrin-mediated endocytosis rather than other pathway. Particle size has been reported to determine the route of cellular entry. However, the size dependence for uptake by caveola or clathrin-coated vesicles was found to vary with the nature of the particles, the cell types studied, or both [46]. In this study, temperature-responsive liposomes could increase the particle size by aggregation and dehydrated their surfaces with raising temperature. Then the microtubules may mostly be involved in the uptake of larger particles.

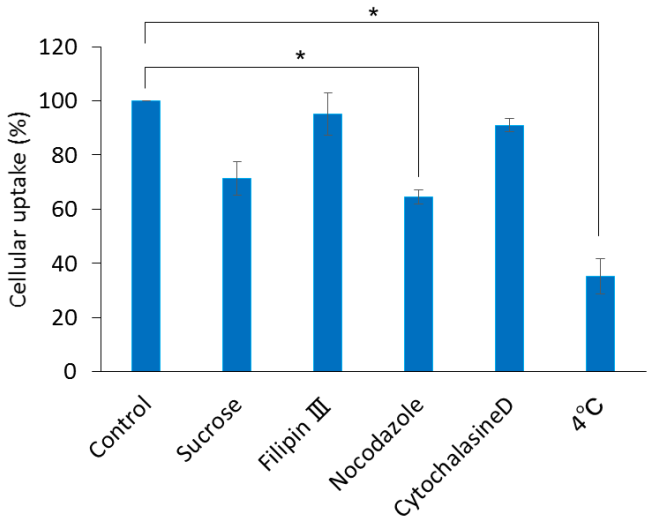

Figure 3. 7. HeLa cells were pretreated with sucrose, filipin III, nocodazole, and cytochalasine D at 37 °C for 1 h or incubated at 4 °C for 1 h. Control was not pretreated. Then, cellular association of rhodamine-labeled and P(NIPAAm-*co*-DMAPAAm) liposomes was examined in pretreated HeLa cells following incubation at 40 °C or 4 °C for 30 min. Mean ± standard deviation (S.D., n = 3); *$p < 0.05$ vs control and **$p < 0.01$ vs control.

The cellular uptake of the PNIPAAm-copolymer and PNIPAAm-chitosan-modified magnetite nanoparticles [27], as well as the PNIPAAm-PAMPTMA polymer complex with

siRNA [28], was reported following heating. However, the dehydration-induced cellular uptake of the PNIPAAm-copolymer-modified liposomes has not been clearly reported. The difference of cellular uptake between nanoparticles and liposomes may be attributable to the particle size change with temperature control. The self-assembled PNIPAAm-copolymer micellar and solid nanoparticles covered with the copolymer are hardly or slowly aggregated by the heat-induced dehydration of copolymer and, therefore, retain their small particle size, resulting in cellular uptake [24]. Moreover, liposomes modified with PNIPAAm-copolymers exhibited a fast aggregation induced by raising temperature to induce a rapid drug release, resulting in a lower cellular uptake than nanoparticles. In this study, my temperature-responsive liposomes induced aggregation and release at the copolymer LCST and exhibited a slow rate of aggregation. The hydrated layer of the PNIPAAm-copolymer on the unstable DOPE-rich cationic liposomes increased the colloidal stability and lower cytotoxicity of the liposomes, comparable to PEGylated liposomes. The dehydration of temperature-responsive liposomes increased aggregation, destabilization, and release of content. The cellular uptake was on a delicate balance between the aggregation and release by the temperature-responsive liposomes. Therefore, considering these factors in designing PNIPAAm-copolymers could increase the intracellular delivery of the modified liposomes.

The non-modified liposomes showed significantly cytotoxicity, but the PEGylated and temperature-responsive liposomes did not observed at 37 °C or 40 °C. It was surprising to note that the majority of the liposome-entrapped CF was associated with the cell surface, confirming that the elevated temperatures used for these experiments were not detrimental to the cells based on a cell viability assay (**Figure 3. 7**).

In this study, temperature-responsive liposomes increased its particle size by aggregation and their surfaces were dehydrated by raising temperature. Microtubules

may mostly be involved in the uptake of larger particles. Therefore, these surface tunable polymer-modified liposomes could have great potential as intracellular drug delivery carriers for various products. However, further studies are needed to elucidate the cellular uptake and intracellular trafficking mechanisms involved in these processes and the contributions of the polymer characteristics of the liposomes.

3.4. Conclusion

To improve the cellular uptake and release about temperature-responsive liposomes, I synthesized P(NIPAAm-*co*-DMAPAAm)-DOPE and investigated its physicochemical properties by using it to modify DOTAP/DOPE liposomes as temperature-responsive liposomes. Below the LCST of this copolymer, the temperature-responsive liposomes had hydrated surface and aqueous layer similar to the PEGylated liposomes. Furthermore, the temperature-responsive liposomes were stable and hardly release their encapsulated content below the LCST. Above the LCST of this copolymer, the temperature-responsive liposomes induced cellular uptake and released the encupsulated content into the cytosol more than the PEGylated liposomes. To the best of our knowledge, this is the first study to report that the dehydration of temperature-responsive polymer-modified liposomes induced faster cellular uptake and release than other liposomes. The temperature-triggered tunable surface properties of temperature-responsive P(NIPAAm-*co*-DMAPAAm)-modified liposomes could be highly applicable to intracellular drug delivery carriers.

4. siRNA and plasmid transfection by temperature-responsive liposomes

4. 1. Materials

4.1.1. Materials

N-[1-(2,3-Dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl-sulfate (DOTAP) ・・・・・・・・・・・・・・・・・・・・・Avanti Polar Lipids Inc. (Alabaster, AL, USA)

L-α-phosphatidylethanolamine, dioleoyl (DOPE)・・・・・・・・・・・・・・・・・・・・・・・・・・・・・・・・・・・Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

N-[methoxy (polyethylene glycol)2000]-distearoyl phosphatidylethanolamine (PEG-DSPE)・・・・・・・・・・・・・・・・・・Avanti Polar Lipids Inc. (Alabaster, AL, USA)

5(6)-carboxyfluorescein (CF)・・・・・・・・・・ Sigma-Aldrich Corp. (St. Louis, MO, USA)

Minimum essential medium (MEM)・・・・Thermo Fisher Scientific (Waltham, CA, USA)

High glucose DMEM・・・・・・・・・・・Thermo Fisher Scientific (Waltham, CA, USA)

Bovine serum (FBS)・・・・・・・・・・・・・・・・・・・・・・・・・Biosera (Sussex, UK)

Penicillin-Streptomycin Glutamine・・・・・Thermo Fisher Scientific (Waltham, CA, USA)

MEM Non-Essential Amino Acids (NEAA)・・・・・・・・・・Thermo Fisher Scientific (Waltham, CA, USA)

G-418 sulfate・・・・・・・・・・・・・Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

Dulbecco's phosphate-buffered salines (PBS)・・・・・・・・・Thermo Fisher Scientific (Waltham, CA, USA)

0.05 w/v% Trypsin-0.53 mmol/L EDTA・4Na・・Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

Water-soluble tetrazolium (WST)-8・・・・・・・・・・・・・・(Dojindo, Kumamoto, Japan)

Sucrose・・・・・・・・・・・・・・・・Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

Cytochalasin D・・・・・・・・・・・・Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

Nocodazole・・・・・・・・・・・・・・Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

Filipin III・・・・・・・・・・・・・・・・・・・Cayman Chemical (Ann Arbor, MI, USA)

Amiloride・・・・・・・・・・・・・・・・・・・・・・・Funakoshi Co., Ltd. (Tokyo, Japan)

Lipofectamine RNAiMAX (RNAiMAX)・・・・・・・・・ Invitrogen, Corp. (Carlsbad, CA)

Lipofectamine 2000・・・・・・・・・・・・・・・・・ Invitrogen, Corp. (Carlsbad, CA)

Lysis buffer・・・・・・・・・・・・・・・・・・・・・・・Promega (Madison, WI, USA)

Luciferase assay system Pica Gene・・・・・・・・・Toyo Ink Mfg. Co. Ltd. (Tokyo, Japan)

BCA reagent・・・・・・・・・・・・・・・・・・・・・・・Pierce (Rockford, IL, USA)

4.1.2. pDNA and siRNA.

siRNAs targeting firefly luciferase nucleotides (Luc siRNA) were synthesized by Sigma Genosys (Tokyo, Japan). The anti-Luc siRNA (antisense sequence: 5′-UUAGACACGAACACCACGGTT-3′, sense sequence: 5′-CCGUGGUGUUCGUGUCUAATT-3′, anti-sense sequence: 5′-UUAGACACGAACACCACGGTT-3′) and BLOCK-iT™ Alexa Fluor® Red Fluorescent Control siRNA (AF-siRNA) were purchased from Invitrogen (Life Technologies, Carlsbad, CA). The anti-green fluorescent protein (GFP) siRNA (sense sequence: 5′-CCGUGGUGUUCGUGUCUAATT-3′, anti-sense sequence: 5′-UUAGACACGAACACCACGGTT-3′) was obtained from Sigma Genosys (Tokyo, Japan). Plasmid DNA (pDNA, MSCV-IRES-GFP) was kindly provided by Dr. Megumi Tago (Keio University, Tokyo, Japan).

4.2. Methods

4.2.1. Preparation of Liposomes Carriers and Lipoplexes.

All liposomes in sterile deionized water were prepared using a thin-film hydration method. The polymer-modified liposomes were prepared with DOTAP, DOPE, and P(NIPAAm-*co*-DMAPAAm)-DOPE or PEG-DSPE at a fixed molar ratio of 30/65/5 in chloroform solution, where not otherwise specified, and the solutions in the flasks were subsequently evaporated. P(NIPAAm-*co*-DMAPAAm)-DOPE was used for temperature-responsive liposomes and PEG-DSPE for PEGylated liposomes. The non-modified liposomes were prepared with DOTAP and DOPE (1:1 molar ratio). The liposomes suspension was extruded 11 times through a polycarbonate membrane with 100 nm pore diameter using mini-extruder (Avanti Polar Lipids, Inc., Abaster, AL).

To prepare liposomes complexed with siRNA (lipoplexes), each liposome suspension (20 mg total lipid/mL water) and siRNA aqueous solution (200 μM stock concentration) were separately diluted with cell culture medium, mixed at a charge ratio (+/–) of 5/1 by vortex-mixing for 10 sec, and left for 30 min at room temperature. The lipoplexes, with a final concentration of 50 nM siRNA or 30 nM AF-siRNA, were used immediately after preparation. The theoretical charge ratio (+/-) of cationic liposomes to siRNA was calculated as the molar ratio of DOTAP to siRNA phosphate. Lipofectamine RNAiMAX (RNAiMAX) and Lipofectamine 2000 were used as positive controls for siRNA and pDNA transfections, respectively, and transfection procedures were performed in accordance with the manufacturer's instructions.

4.2.2. Particle Size and Zeta Potential Measurements about lipoplexes.

The mean particle size and zeta potential of the liposomes and lipoplexes were measured using dynamic light scattering with a Zetasizer Nano-ZS (Malvern, Inst. Ltd. Malvern, UK) and an electrophoretic light scattering ELSZK-2 KOP apparatus (Otsuka

Electronics Co., Ltd., Osaka, Japan), respectively, at 25 °C in water and at 30, 37, and 40 °C in DMEM.

4.2.3. Cell Culture.

Human epithelioid cervix carcinoma HeLa cells stably expressing a firefly luciferase (HeLa-Luc) were donated by Dr. Kenji Yamato (Tsukuba University, Tsukuba, Japan). HeLa-Luc cells were cultured as subconfluent monolayers in 75 cm^2 culture flasks with vent caps, using high-glucose DMEM supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL G-418 sulfate. HeLa cells (RIKEN BRC Cell Bank, Ibaraki, Japan) were also cultured as subconfluent monolayers in 75 cm^2 culture flasks with vent caps using MEM supplemented with 10% FBS, 50 units/mL penicillin and 50 μg/mL streptomycin, at 37 °C in a humidified incubator containing 5% CO_2. Subconfluent cells were dissociated using 0.05 w/v% trypsin 0.53 mmol/L EDTA·4Na solution and cultured in new flasks for 3-4 days.

4.2.4. Determination of Cellular Uptake about lipoplexes by Flow Cytometry and Fluorescence Microscopy.

To detect the cellular association with the lipoplexes, HeLa cells (5.0×10^4 cells/well) were added to 6-well culture plates 24 h prior to each experiment. Lipoplexes with AF-siRNA were diluted in culture medium supplemented with 10% FBS, and added to each well to achieve a final concentration of 30 pmol/mL AF-siRNA. After 2 h incubation at 30, 37, and 40 °C, plates were washed twice with 1 ml phosphate buffered saline (PBS) to remove any unbound lipoplexes and cells were harvested with trypsin/EDTA. The cells were further washed three times by centrifugation and resuspended in PBS, and their cell-associated fluorescence was determined on a FACSCalibur flow cytometer (BD™

LSR II, Becton Dickinson, San Jose, CA, USA) equipped with a PE filter. For observation by fluorescence microscopy, the cells incubated with the lipoplexes for 2 h were washed twice with PBS, and fresh PBS was added to the wells. Examinations were performed with a BZ-9000 all in one fluorescence microscope (BZ-9000, Keyence Corp., Osaka, Japan).

4.2.5. Evaluation of Transfection Efficiency.

To construct transitory GFP-expressing HeLa cells, 5.0×10^4 cells/well were added to 6-well culture plates 24 h prior to each experiment, and pDNA transfection efficiency by each carrier was examined. pDNA encoding a GFP gene complexed with the temperature-responsive liposomes at a charge ratio (+/-) of 5, or Lipofectamine 2000 prepared according to the manufacturer's specifications, were diluted in medium supplemented with 10% FBS, then added to each well and incubated for 48 h at 37 °C. The final concentration of pDNA per well was 5 μg/mL. Following processes for flow cytometer as described above, the percentage of GFP-expressing cells was determined using a FACSCalibur flow cytometer equipped with a filter appropriate for the detection of 530/30 bandpass filter. To determine GFP gene expression by fluorescence microscopy, the cells incubated with the lipoplexes for 48 h were washed twice with PBS, and fresh PBS was added to the wells. Examinations were performed as described above.

4.2.6. Estimation of Gene Silencing.

The gene silencing capabilities of each carrier were investigated in both HeLa-Luc cells and transitory GFP-expressing HeLa cells. HeLa-Luc cells (5.0×10^4 cells/well) were added to 6-well culture plates 24 h prior to each experiment. Luc siRNA and Cont siRNA lipoplexes were diluted in 1 ml medium supplemented with 10% FBS, then added to each

well to achieve a final concentration of 50 nM Luc siRNA or Cont siRNA, and incubated for 48 h at 30, 37, or 40 °C. The transfected cells were then washed twice with PBS and harvested by treatment with a lysis buffer. Luciferase activity in the cell lysates was measured as counts per second (cps)/g protein using a luciferase assay system and BCA reagent. Luciferase activity (%) was calculated relative to the luciferase activity (cps/g protein) of untreated cells.

To co-transfect siRNA, the cells were first transfected with GFP pDNA lipoplexes as described above, and incubated at 37 °C. 4 hours later, the supernatants were discarded and the cells were washed with PBS. The cells were then transfected a second time with 50 nM GFP siRNA using temperature-responsive liposomes or RNAiMAX and incubated further for 44 h at 37 °C. The GFP expression of the cells was determined by flow cytometry and fluorescence microscopy as described above. The resulting fluorescence activity (RFU) was corrected against the amount of protein (micro g) to obtain normalized GFP expression. Silencing activity (%) was calculated as ((1-relative to GFP expression (RFU/g protein) of untreated cells) ×100).

4.2.7. Cell Viability Assessment.

The cytotoxic potential of the carriers was evaluated by WST-8 assay. HeLa cells (5.0×10^3 cells/well) were added to 96-well culture plates 24 h prior to each experiment. Lipoplexes with Luc siRNA were diluted in medium supplemented with 10% FBS, and added to each well to achieve a final concentration of 50 nM Luc siRNA. After 48 h incubation at 37 or 40 °C, 10 μL WST-8 was applied to each well and incubated at 37 °C for 1 h. The number of viable cells was determined by measuring absorbance at 450 nm using a microplate reader (TECAN infinite M-1000, Tecan Japan Co., Kanagawa, Japan). Cell viability was expressed as a percentage of the untreated control cells.

4.2.8. Uptake after Treatment with Endocytosis Inhibitors.

HeLa cells (5.0×10^4 cells/well) were added to 6-well culture plates 24 h prior to each experiment. Pretreatment conditions were either incubation at 4 °C for 1 h, or with serum-free DMEM containing cytochalasin D (5 μg/mL), nocodazole (3 μg/mL), sucrose (69 mg/mL), amiloride (0.7 μg/mL), or filipin III (2 μg/mL) at 37 °C for 1 h. Medium was then replaced with 1 ml medium containing 10% FBS, each inhibitor, and the temperature-responsive and non-modified lipoplexes with 30 pmol AF-siRNA. The cells were further incubated at 40 °C and 37 °C for 2 h. Cells were then washed twice with PBS, harvested using trypsin/EDTA, and processed for flow cytometry as described above.

4.2.9. Statistical Analysis.

The Student's non-paired *t*-test was used to compare the normally distributed values between the groups. $p < 0.05$ was considered statistically significant.

4.3. Results and discussion

4.3.1. Selection of Temperature-responsive Lipoplexes.

The formulation of temperature-responsive liposomes and lipoplexes was examined by measurement of gene silencing activity after transfection of Luc siRNA using each carrier into HeLa-Luc cells.

To determine the optimum mixture ratio of DOTAP and DOPE in the liposomes for transfection, DOTAP/DOPE liposomes (molar ratio, 1:1, 4:6, and 3:7) modified with 5%

P(NIPAAm-*co*-DMAPAAm)-DOPE polymer were prepared, complexed with siRNA at a charge ratio (+/−) of 5/1, and evaluated. Gene silencing activity seemed to increase with increasing DOPE ratio, but no significant difference in gene silencing activity among the lipoplexes was observed (**Figure 4. 1a**). Since cationic DOTAP is cytotoxic [47], I selected a lower DOTAP ratio, choosing liposomes with a molar ratio of DOTAP/DOPE = 3:7.

Hydrophilic polymer concentration on liposomes is important for interaction with the cell membrane, as with PEGylation [9]. When the polymer concentration was increased from 5 to 10 mol% in DOTAP/DOPE = 3/7 liposomes, gene silencing activity was significantly reduced (**Figure 4. 1b**). It may be that the LCST of P(NIPAAm-*co*-DMAPAAm) was reduced by increasing the polymer concentration. This finding indicates that 5 mol% polymer modification on the liposome is a suitable density for a siRNA carrier. Thus, the optimum formulation of the temperature-responsive liposomes was determined as DOTAP, DOPE, and PNIPAAm-*co*-DMAPAAm)-DOPE (molar ratio, 30:65:5).

To determine an appropriate charge ratio, temperature-responsive lipoplexes at charge ratios (+/−) of 5/1, 10/1, and 20/1 were prepared and evaluated. No significant differences were observed among them (**Figure 4. 1c**). DOTAP/DOPE (molar ratio, 1:1) lipoplexes were reported to increase gene silencing activity with increasing charge ratios (+/−) up to 20 [48]. Considering the increase in cytotoxicity caused by increased DOTAP, a charge ratio (+/−) of 5/1 of the lipoplexes with each carrier was fixed.

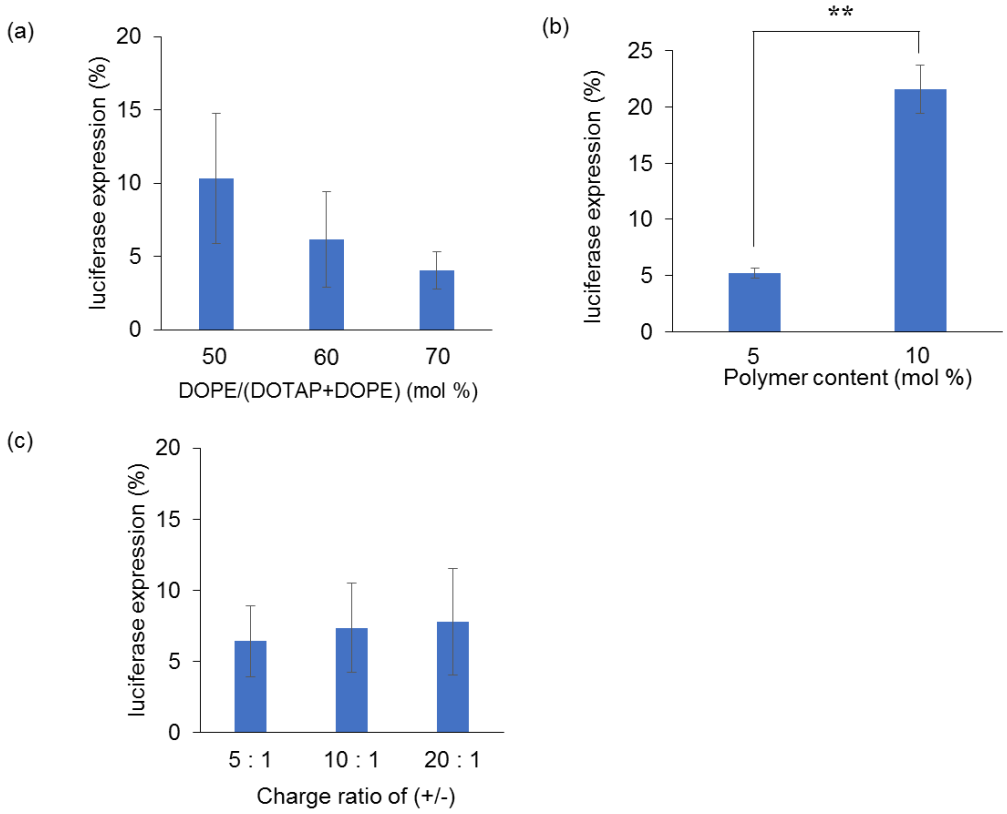

Figure 4. 1. Effect of a mixture ratio of DOTAP and DOPE (a), polymer amounts (b) and charge ratios (+/–) (c) of the liposomes complexed with Luc siRNA on gene silencing activity, following incubated with HeLa-Luc cells for 48 h at 37 °C. Mean±S.D. (n=3); **p < 0.01.

4.3.2. Size and zeta potential of the lipoplexes.

The mean diameters of the temperature-responsive, PEGylated and non-modified lipoplexes were 144-192 nm, with low PDI values at 25 °C in water (**Table 4. 1**). The size of lipoplexes is generally greater than the liposome alone. However, the size of temperature-responsive, PEGylated lipoplexes was not increased, and that of non-modified lipoplex was slightly increased, compared to that of the liposomes, 141-161 nm, suggesting siRNA had been entrapped in the liposomes. This is supported by the report that DOTAP/DOPE showed a multilamellar organization with closely packed lipid bilayers and siRNA stacked between the bilayers [48].

Table 4. 1. Particle size, polydispersity index (PDI) and zeta potential of liposomes and siRNA lipoplexes in water at 25 °C.

	Liposomes			Lipoplexes [1)]		
	Size (nm)	PDI [2)]	ζ potential (mV)	Size (nm)	PDI	ζ potential (mV)
Non-modified liposome	152.1± 0.50	0.19	61.7± 3.6	191.9± 1.59	0.15	37.2± 0.2
PEGylated liposome	161.2± 1.48	0.24	62.8± 3.2	165.3± 0.47	0.27	37.8± 0.4
Temperature-responsive liposome	141.1± 0.27	0.14	50.0± 0.9	144.4± 0.44	0.12	36.5± 0.8

Mean±SD, n=3; 1) at charge ratio (+/-) of cationic liposomes/siRNA=5/1, 2) mean value.

Zeta potential of the temperature-responsive lipoplexes was found to be reduced to 36.5 mV, compared to that of the liposomes, 50.0 mV, suggesting that negatively charged siRNA was adsorbed on the cationic liposomal surface (**Table 4. 1**). Moreover, zeta potential of PEGylated lipoplexes decreased to 37.8 mV compared to that of the liposomes, 62.8 mV, and that of non-modified lipoplexes was reduced to 37.2 mV from 61.7 mV. All three kinds of lipoplexes dropped to similar zeta-potential values, although the corresponding liposomes had different zeta-potential values, suggesting that a portion of siRNA was adsorbed on the cationic liposomal surface through electrostatic interaction although the polymer covered two kinds of liposomes.

P(NIPAAm-*co*-DMAPAAm) exhibited LCST at approximately 40 °C in water, determined using both 50% change of transmittance and the peak temperature of DSC (data not shown). Because the LCST of the temperature-responsive polymer was affected by electrolytes and proteins in the medium, the initiation temperatures aggregation of the temperature-responsive liposomes in PBS and DMEM were 39 and 37 °C, respectively, lower than that of the copolymer LCST measured in water (**Figure 4. 2**).

When the temperature increased above this value, the temperature-responsive liposomal aggregates in DMEM increased in size sharply, more so than those in PBS.

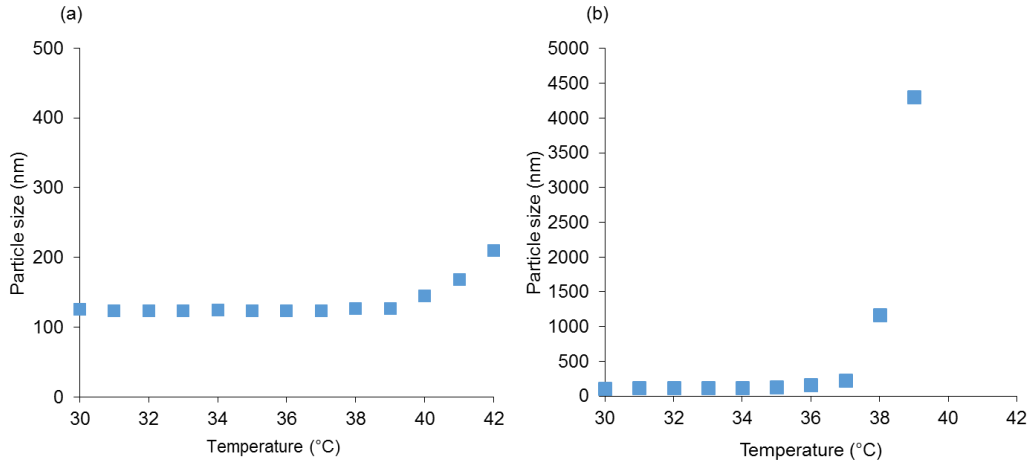

Figure 4. 2. Change in particle size of temperature-responsive liposomes suspension in phosphate-buffered saline (PBS) (a) and Dulbecco's modified Eagle's medium (DMEM) (b) with increasing temperature.

Similar to the liposomes at 30, 37, and 40 °C in DMEM, the particle size of the temperature-responsive lipoplexes increased significantly from 106 nm at 30 °C to 149 nm at 40 °C ($p < 0.05$), while that of non-modified liposomes did not change significantly from 193 nm at 30 °C to 194 nm at 40 °C (**Table 4. 2**). It is speculated that during incubation with cells in DMEM supplemented with 10% FBS, the aggregation initiation temperature of the lipoplexes was reduced and the size of the aggregates quickly increased at 37 °C.

Table 4. 2. Particle size and polydispersity index (PDI) of liposomes and siRNA lipoplexes in DMEM at each temperature.

	30 °C		37 °C		40 °C	
	Size(nm)	PDI	Size(nm)	PDI	Size(nm)	PDI
Non-modified lipoplex*	192.7±0.64	0.12	191.7±2.33	0.13	194.1±1.89	0.07
Temperature-responsive lipoplex*	105.5±3.78	0.14	107.7±3.71	0.15	148.8±2.37	0.32

The LCST of temperature-responsive polymer was 40.2 °C in water determined by transmittance changes. Lipoplexes were prepared at charge ratio (+/-) of cationic liposome/siRNA=5/1.

4.3.3. Cellular Association of siRNA through Carriers.

Association of the lipoplexes with HeLa cells was determined at 37 °C using AF-siRNA by flow cytometer and fluorescence microscopy. Intracellular tracking of siRNA maybe is the same of the liposome. First, the effect of incubation time on association of the temperature-responsive lipoplexes was examined at 37 °C by flow cytometer (data not shown). The association of the lipoplexes increased with increasing incubation time, and seemed to be saturated during 4 h incubation. Therefore, cellular association of the lipoplexes composed of various carriers was examined after 2 h incubation at 37 °C. By flow cytometer, since a decrease in fluorescence intensity indicates decreased cellular association of siRNA by each carrier, the lipoplex association at 37 °C showed the following order: temperature-responsive liposomes (1.14) = RNAiMAX (1) >> PEGylated liposomes (0.07) (**Figure 4. 3**). The number in parenthesis represents the fluorescence ratio on the basis of RNAiMAX. It indicates that the temperature-responsive liposome carrier achieved higher cellular uptake of siRNA, similar to RNAiMAX, but PEGylated liposomes did not. However, non-modified liposomes showed similar fluorescence intensity values to the temperature-responsive liposome with a large deviation. This

might be due to loosely bound lipoplex [49].

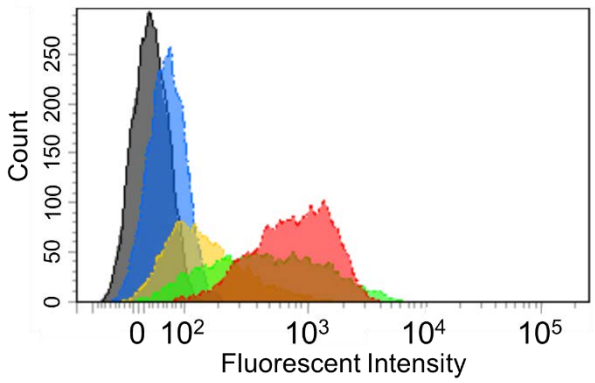

Figure 4. 3. Cellular association of Alexa fluor-555 labeled siRNA lipoplex with PEGylated (blue), non-modified (yellow), Lipofectamine RNAiMAX (green), and temperature-response liposome (red) incubated with HeLa cells and the untreated HeLa cells (black) for 2 hours at 37 °C.

Cellular association of the lipoplex was further investigated by fluorescence microscopy. The level of cell-associated fluorescence at 37 °C showed the following order: temperature-responsive liposomes = RNAiMAX > non-modified liposomes >>PEGylated liposomes, showing bright red fluorescence (**Figure 4. 4a**). Utilizing the difference in thermal behavior between non-modified and temperature-responsive liposomes, the cellular uptake after incubation at 30 and 40 °C was further observed under higher magnification (**Figure 4. 4b**). At 30 °C, the temperature-responsive lipoplexes showed lower cellular association similar to that of non-modified liposomes, exhibiting red fluorescent dots of AF-siRNA lipoplex surrounding the cell membrane. However, at 40 °C, many red fluorescent dots representing the temperature-responsive lipoplexes were observed in the cytoplasm, more so than that of non-modified lipoplexes, indicating that the temperature-responsive lipoplexes were taken up into the cells in higher numbers than non-modified lipoplexes.

Positively charged non-modified lipoplexes may interact strongly with negatively

charged cell membranes, and lipoplexes adsorbed onto cell surfaces are not easily internalized by the cells [24]. Meanwhile, the PEGylated liposomes retained the hydrated layer from 30 and 40 °C observed in a previous study [50], which disturbed cell interactions and decreased uptake of the lipoplexes [6, 7]. Although the temperature-responsive liposomes may still have a partly hydrated layer at 37 °C, dehydrated globule polymers on the liposomes were likely to promote cellular uptake [36]. Because the pKa of P(NIPAAm-*co*-DMAPAAm) was 8.2, the tertiary amine moiety of DMAPAAm at pH 7.4 was more protonated, while DOTAP possesses the quaternary ammonium cation, which is pH-independent. The positively charged globule polymer may contribute to the interaction of temperature-responsive liposomes with cell membranes, more so than non-modified lipoplexes.

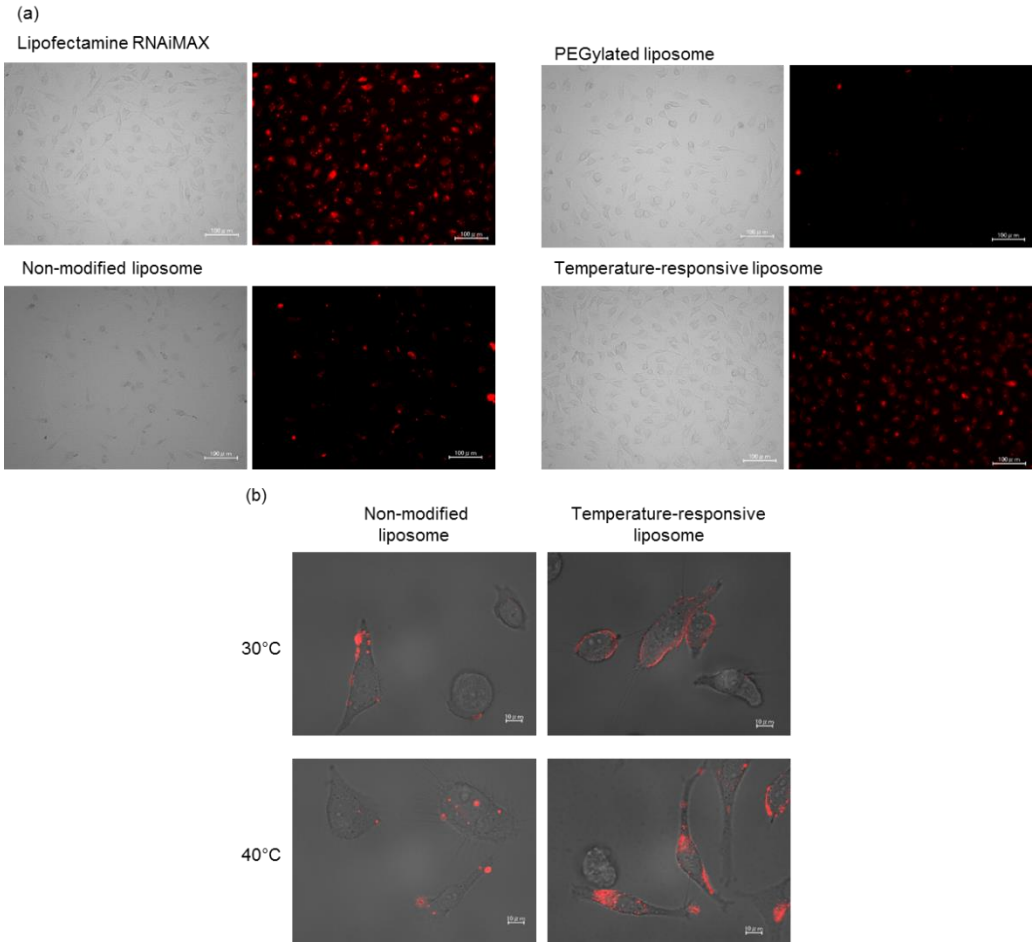

Figure 4. 4. Cellular association of Alexa fluor 555-labeled siRNA lipoplexes using each carriers incubated with HeLa cells for 2 h at 37 °C (a), and 30 and 40 °C (b), observed by fluorescence microscopy. Left panel is bright field and right panel is fluorescence (a). Scale bar represents 10 μm (a) and 100 μm (b).

4.3.4. Transfection Efficiency of GFP pDNA.

To assess the co-transfection of GFP siRNA, transiently expressing-GFP HeLa cells were constructed. Transfection efficiency of GFP pDNA using the temperature-responsive and PEGylated liposomes, or Lipofectamine 2000, was assessed by fluorescence microscopy and flow cytometry. By fluorescence microscope, the GFP expression (green) in the cells showed similar results when using temperature-responsive liposomes and Lipofectamine 2000, but not when using PEGylated liposomes (**Figure 4. 5a**). Consistently, by flow cytometry, the GFP transfection efficiency of the

temperature-responsive lipoplex (26.7%) was not significantly different to that of Lipofectamine 2000 (24.1%, $p > 0.05$) (**Figure 4. 5b**). The GFP transfection efficiency of Lipofectamine 2000 in HeLa cells was similar to the reported figure of 31% [51]. This finding indicates that the temperature-responsive liposomes could deliver pDNA into the nucleus well, and could also be effective as a pDNA transfection carrier.

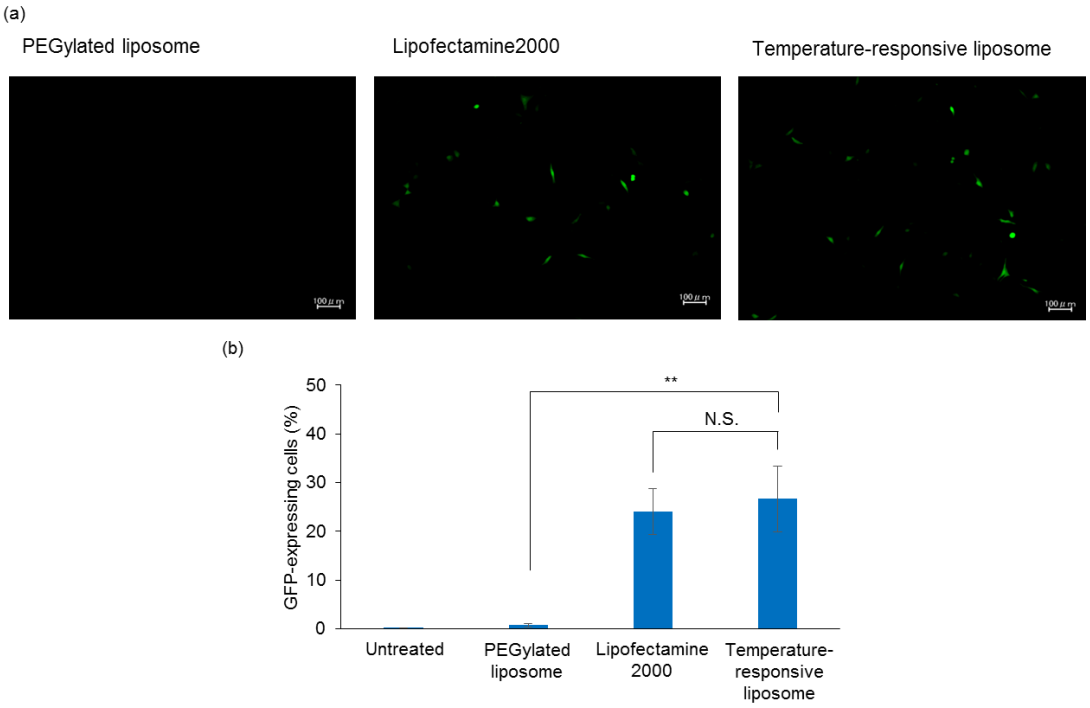

Figure 4. 5. GFP transfection efficiency of GFP pDNA complex with each carriers following 48 h incubation at 37 °C. GFP expression in HeLa cells was observed by fluorescence microscope (a) and flow cytometer (b). Transfected cells appear green GFP expression; the scale bar represents 100 μm. Mean±S.D. (n=3); **$p < 0.01$, $p>0.05$; N.S., nonsignificant.

4.3.5. Gene silencing activity about GFP.

In order to determine the silencing activity of carrier on delivery against GFP using fluorescence microscopy and flow cytometry.

Gene silencing activity was assessed by co-transfection of GFP siRNA with each carrier

into HeLa cells transiently expressing a GFP gene (protein, mRNA) at 37 °C. By fluorescence microscopy, GFP fluorescence in the cells was not observed when using RNAiMAX, or non-modified or temperature-responsive liposomes, but was observed when using PEGylated liposomes (data not shown). This indicates that RNAiMAX and temperature-responsive liposomes were effective carriers of siRNA. By flow cytometry, the temperature-responsive lipoplexes showed effective silencing activity (15.5±5.1%), similar to that of RNAiMAX (8.8±2.3%, $p > 0.05$) at 37 °C (**Figure 4. 6**).

The temperature-responsive liposomes strongly enhanced gene silencing efficiencies after co-transfection and direct transfection of siRNA, similar to RNAiMAX at 37 °C, indicating targeting of luciferase and GFP mRNA, respectively. This proves that the temperature-responsive lipoplexes taken up by the cells released siRNA into the cytosol. From the literature, DOTAP/DOPE lipoplexes showed high gene knockdown was shown at approximately 20-50 mol% DOPE, which decreased above 50% DOPE [48]. In this study, non-modified liposomes composed of 50 mol% DOPE were considered to have the most effective gene silencing activity of a series of DOTAP/DOPE liposomes and, therefore, have often been used as gene carriers. The gene silencing activity of the non-modified lipoplexes in this study was similar to that previously reported in HeLa cells at 37 °C [52].

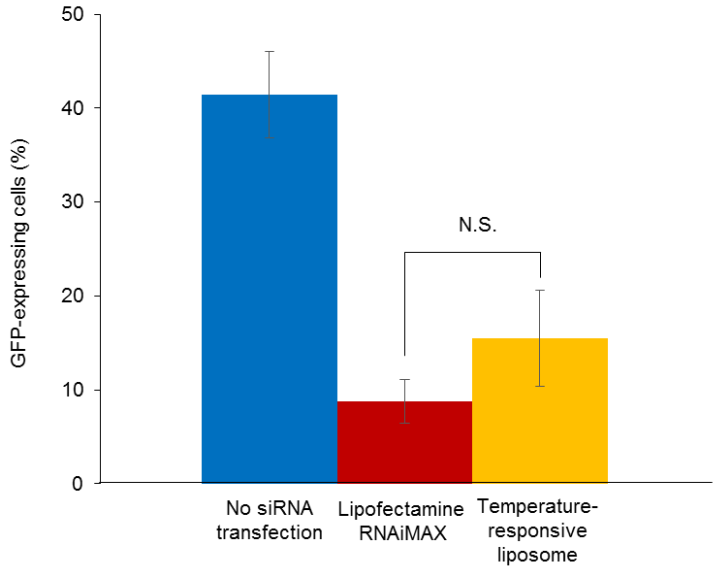

Figure 4. 6. Gene silencing activity through co-transfection of GFP siRNA lipoplexes into GFP pDNA transfected HeLa cells. GFP pDNA was transfected using temperature-responsive liposomes for 4 h incubation, and then GFP siRNA was transfected using each carriers or the cells were untreated following 44 h incubation at 37 °C, observed by flow cytometry. Mean±S.D. (n=3); $p > 0.05$; N.S., nonsignificant.

4.3.6. Gene silencing activity about luciferase.

Next, gene-silencing activity was assessed by transfection of Luc siRNA using each carrier into HeLa-Luc cells at 30, 37, and 40 °C by flow cytometry. Untreated cells were used as a control of 0% gene silencing. The cells treated with naked siRNA showed silencing activity of 96%, 87%, and 92% at 30, 37, and 40 °C, respectively (**Figure 4. 7**), and naked Cont. siRNA, and Cont. siRNA lipoplexes using RNAiMAX, non-modified, PEGylated, and temperature-responsive liposomes showed 114%, 104%, 87%, 98%, and 101% at 40 °C, respectively (data not shown). At 30 °C, temperature-responsive liposomes showed significantly lower gene silencing activity than RNAiMAX and non-modified liposomes, similar to that of PEGylated liposomes. The gene silencing activity for each carrier decreased in the following order: RNAiMAX (94%) > non-modified liposomes (72%) >> temperature-responsive liposomes (25%) ≈ PEGylated liposomes

(15%) compared to the control. On the contrary, at 37 °C, the temperature-responsive liposomes showed increased gene silencing activity, similar to that of RNAiMAX, and significantly higher than that of non-modified liposomes. The gene silencing activity for each carrier decreased in the following order: RNAiMAX (98%) ≈ temperature-responsive liposomes (95%) > non-modified liposomes (77%) >> PEGylated liposomes (0%) compared to the control. This finding is consistent with the report that PEGylated liposomes modified with 5 mol% PEG2000 completely abolished siRNA-mediated gene silencing in vitro [33]. At 40 °C, however, the temperature-responsive liposomes did not increase gene silencing activity compared to that shown at 37 °C. Cell viability at 40 °C was similar to that at 37 °C (**Figure 4. 8**), and the cellular uptake amounts of the lipoplexes at 37 and 40 °C may be finally close over 4 h incubation. Long incubation periods (48 h) with lipoplexes are required for gene knockdown, which may be one of the reasons why similar gene silencing activities at 37 and 40 °C were observed.

The relative luciferase gene silencing activities of the temperature-responsive lipoplexes at 30, 37, and 40 °C were 25%, 95%, and 95%, respectively, likely due to a thermally responsive gene-silencing tendency. Although RNAiMAX and non-modified liposomes showed high gene silencing activity at all tested temperatures, the temperature-responsive liposomes showed significantly higher activity than non-modified liposomes ($p < 0.02$), comparable to RNAiMAX at 37 and 40 °C ($p > 0.05$). Since gene-silencing activity may be due to cytotoxicity causing unspecific silencing, cytotoxicity after gene transfection was examined.

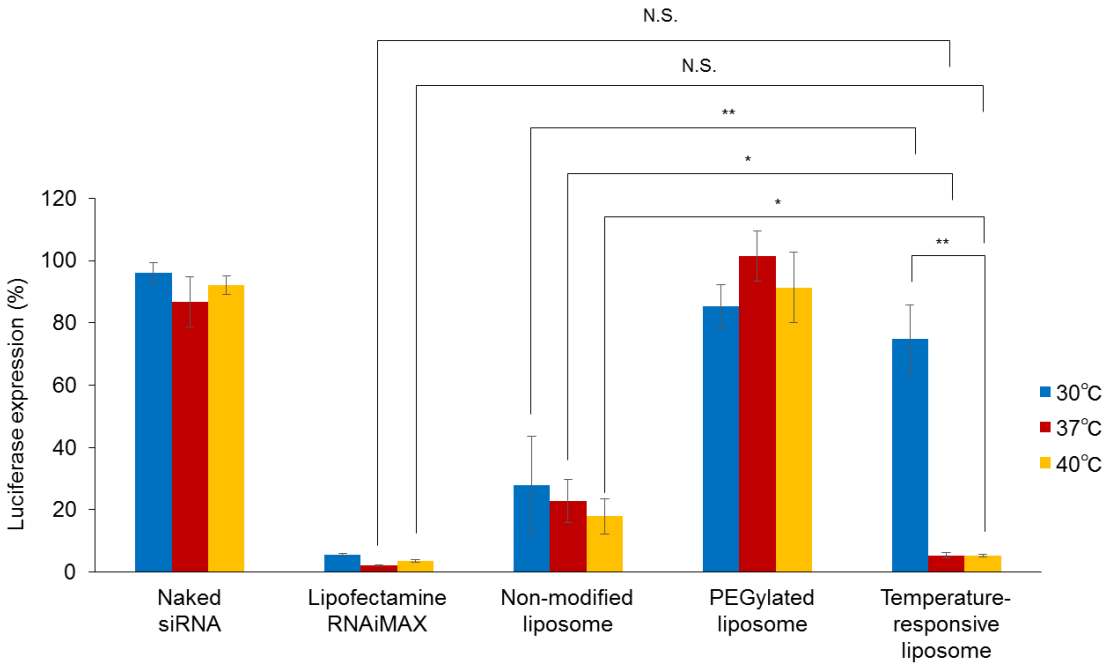

Figure 4. 7. Gene silencing activity of Luc siRNA lipoplexes incubated with HeLa-Luc cells for 48 h at 30, 37 or 40 °C. Mean±S.D. (n=3); *p < 0.02, **p < 0.01, p > 0.05; N.S., nonsignificant.

4.3.7. Cell Viability Assessment after Transfection of siRNA Lipoplexes.

Cell viability after transfection of each lipoplex incubated with HeLa cells for 48 h at 37 and 40 °C was examined. RNAiMAX and non-modified lipoplexes showed significantly low viability at 37 °C, 20% and 45%, respectively, compared with the temperature-responsive lipoplexes (**Figure 4. 8**). However, the temperature-responsive lipoplexes showed high viability (93% and 88%), similar to PEGylated lipoplexes (101% and 90%) at 37 and 40 °C, respectively.

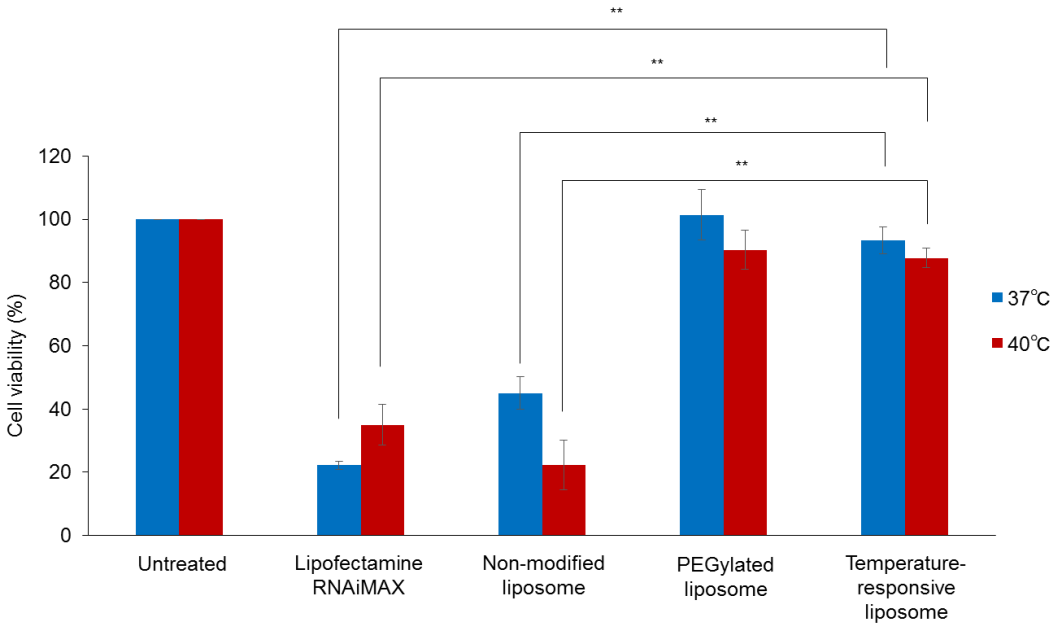

Figure 4. 8. Cell viability incubated with each lipoplexes for 48 h at 37 or 40 °C. Mean±S.D.(n=3); **p < 0.01.

4.3.8. Cellular uptake mechanism of the temperature-responsive lipoplex using inhibitors.

Detailed uptake mechanisms of the temperature-responsive lipoplexes, compared with those of the non-modified lipoplexes, were investigated by utilizing various endocytic inhibitors and flow cytometric analysis. The endocytic inhibitors and concentrations were determined based on previously reported studies [44]. Inhibitor concentrations that showed cell viability values ≥ 80% were selected for this study. As a control, I used cells that had been incubated with each lipoplex in the absence of inhibitors.

The cellular internalization of the temperature-responsive lipoplexes was mainly an energy-dependent process because 71% of the uptake was significantly blocked at 4 °C (**Figure 4. 9**). Clathrin-mediated endocytosis, caveolae-mediated endocytosis, and macropinocytosis are well known mechanisms for the cellular uptake of nanoparticles.

Sucrose for clathrin-mediated endocytosis, filipin III for caveolae-mediated endocytosis, and amiloride for macropinocytosis are inhibitors. The cytoskeleton plays an important role in endocytosis and trafficking of endocytosis vesicles. For this reason, I investigated the role of two of the major constituents of the cytoskeleton structure, F-actin and microtubules, in uptake and internalization. Cytochalasin D can disrupt F-actin polymerization; microtubules are reportedly involved in endocytosis, and nocodazole is a microtubular disruptor [45]. Cytochalasin D and nocodazole are, therefore, inhibitors of actin-based transport microtubular transport, respectively.

The uptake of temperature-responsive lipoplexes was significantly inhibited by nocodazole (38%) ($p < 0.01$, compared with the control) that affects microtubule depolymerization, proposing microtubular transport. Comparatively sucrose exerted a weak inhibition effect (18%) on the internalization of the lipoplexes ($p < 0.05$), but cytochalasin D, filipin III, and amiloride did not show significant effects ($p > 0.05$), indicating weak dependence on the clathrin-mediated endocytosis pathway but not on actin-based transport, the caveolae-mediated endocytosis pathway, or the micropinocytosis pathway.

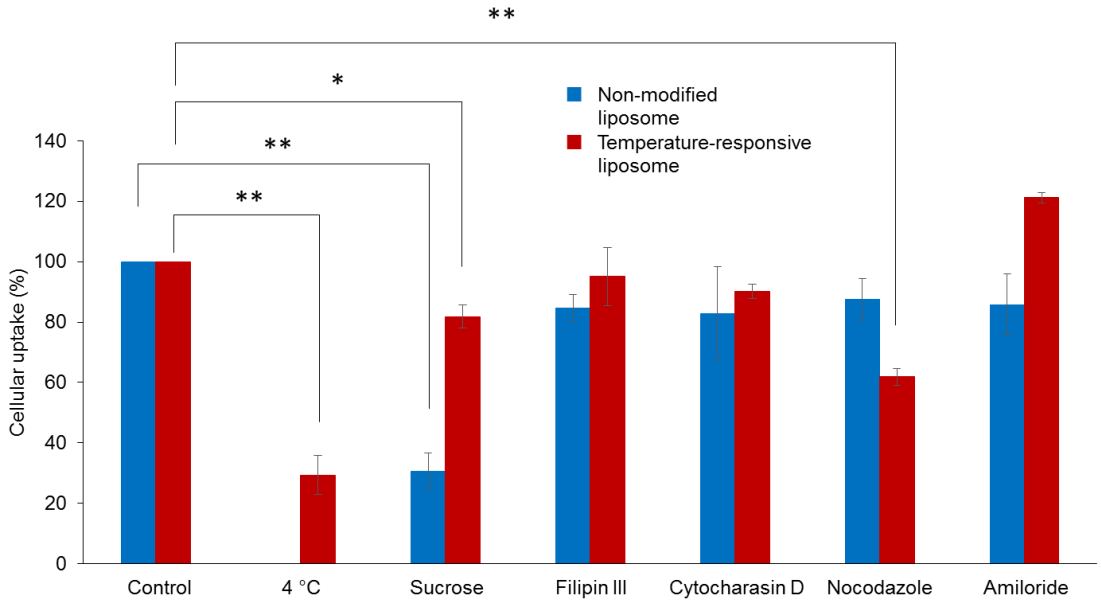

Figure 4. 9. Cellular uptake mechanism of non-modified and temperature-responsive lipoplexes using inhibitors and flow cytometry. HeLa cells were pretreated with each inhibitor at 37 °C for 1 h or incubated at 4 °C for 1 h. Control was not pretreated. Then, cellular association of AF-siRNA lipoplexes using non-modified and temperature-response liposomes were examined in pretreated HeLa cells following incubation at 4 or 40 °C for 2 h. Mean±standard deviation (S.D., n = 3); *p < 0.05, **p < 0.01 vs control.

This finding suggests a bigger role of microtubules, and clathrin-mediated endocytosis to a smaller extent, for lipoplex internalization. However, the uptake of non-modified lipoplexes was significantly inhibited only by sucrose (69%) ($p < 0.01$, compared with control), indicating strong dependence on the clathrin-mediated endocytosis pathway. The clathrin-mediated endocytosis route is employed for the uptake of small nanoparticles, less than 150-200 nm, because of the size limitation of clathrin-coated pits on cellular membranes [53]. This finding is consistent with non-modified lipoplexes retaining a small size at 40 °C while the temperature-responsive lipoplexes, which tended to aggregate, were highly taken up by the microtubule-dependent pathway, which was probably connected with dehydrated polymer-modification and resulting aggregation of the lipoplexes. This is supported by the fact that the corresponding

temperature-responsive liposomes tended to aggregate, showing the same uptake mechanism for the lipoplexes as a previous study [24]. Although labeled compounds were different between rhodamine-labeled lipid for the liposome alone, and Alexa-labeled siRNA for the lipoplex, this finding suggests that at least at early stages of siRNA internalization, siRNA was taken up as the lipoplexes.

While the uptake route of nanoparticles depends on their physical attributes, including particle size, shape, and surface charge, and on the type of cell line [46], lipoplex uptake proceeds mostly by clathrin-mediated endocytosis, as lysosomes are the end point of this internalization pathway [54]. Subsequently, it is believed that lysosomal enzymes degrade siRNA and, therefore, clathrin-mediated endocytosis is not an effective pathway for siRNA delivery. Correspondingly, in this study, non-modified lipoplexes mainly taken up by the clathrin-mediated endocytosis pathway showed less gene silencing activity than the temperature-responsive lipoplexes at 37 °C. On the contrary, the temperature-responsive lipoplexes taken up by the microtubule-dependent pathway, rather than the clathrin-mediated endocytosis pathway, showed higher gene silencing activity. Because nocodazole disrupts microtubules and inhibits the translocation of endosomes and lysosomes [55], the microtubule-dependent pathway inhibited by nocodazole is speculated to be an effective route for siRNA delivery.

Here, I show that higher cellular uptake of siRNA by the temperature-responsive liposomes directly translates into better siRNA gene silencing activity at 37 °C (**Figure 4. 4** and **4. 7**). This indicates that the cellular uptake process is the rate-limiting stage in gene silencing, rather than release of siRNA from the lipoplex into the cytoplasm. This is noteworthy for the design of siRNA carriers.

Cellular uptake and gene silencing activity of the temperature-responsive lipoplexes were higher than those of non-modified lipoplexes at 37 °C, suggesting that dehydrated

polymer chains on the liposomal surface contributed to cellular uptake more than decreased hydration. It can be speculated that partially dehydrated and collapsed polymer bedded on the liposomes, and gradually produced liposome aggregates, enhanced the adhesion to cell membranes, and subsequent induced specific cellular uptake via the microtubule-dependent pathway contributed to high gene silencing activity. This is supported by data from the following reports. Dehydrated elastin-like polypeptide-modified liposomes tended to aggregate, and showed enhanced cellular uptake upon heating [29]. Cellular uptake of carboxylated polystyrene nanoparticles in A549 cells after inhibition with nocodazole was decreased by ~40% relative to control cells, as did clathrin- and caveolae-mediated endocytosis inhibitors when the size of nanoparticles increased to 200 nm [56].

To release siRNA, lipoplexes that are highly stable would not be able to facilitate oligonucleotide delivery at the cell level, which requires lipoplex destabilization [57]. In this study, DOTAP/DOPE (molar ratio, 3:7) control liposomes, were unstable and could not be prepared without PEG-lipid nor NIPAAm-copolymer-DOPE, even below the LCST. When the conformation of a temperature-responsive polymer was changed by heating and not able to recover, the liposomes became unstable and the lipoplexes triggered release of siRNA. This can be explained by a report showing that the shape of DOTAP/DOPE mixtures and DOTAP/DOPE complexed with siRNA changed from a lamellar to an inverted hexagonal (fusogenic) structure, according to the increased ratio of DOPE [58]. Quick dissociation of siRNA from the lipoplexes in the cytoplasm may result from such unstable properties of the DOPE-rich liposome component in the temperature-responsive liposomes. Taken together, siRNA would be rapidly dissociated from the unstable lipoplexes caused by dehydrated polymer and could target mRNA in the cells.

Valuable advantages are the facts that transfection using the temperature-responsive

liposomes were able to be incubated in medium containing serum, which is an easy procedure. In addition, the temperature-responsive liposome as a carrier was able to show both high exogenous DNA expression and siRNA-mediated knockdown efficacy, similar to Lipofectamine 2000 and Lipofectamine RNAiMAX, respectively, commercially available transfection reagents (**Figure 4. 5, 4. 6,** and **4. 7**). Moreover, the temperature-responsive liposomes showed a thermal-dependent tendency in cellular uptake and gene silencing activity (**Figure 4. 4b** and **4. 7**). This was observed because the temperature-responsive liposomes were able to transfect siRNA and express subsequent gene silencing activity without severe cytotoxicity, even at 40 °C, in spite of the positively charged lipoplexes (**Figure 4. 8**). Conversely, PNIPAA-*b*-PAMPTMA diblock copolymer/siRNA complexes were reported to have higher gene silencing activity, and cytotoxicity was dependent on the cloud point of the polymer, shown by using different copolymers [28]. Therefore, NIPAAm copolymer modified cationic liposomes may be suitable dosage forms to deliver siRNA by controlled temperature with lower cytotoxicity.

Temperature-responsive liposomes have impact points which are due to changes in the polymer chain from hydrated and extended soluble state to dehydrated and aggregated insoluble state above the LCST. Near the LCST, hydrated and dehydrated layers on the liposomes coexist. In part, hydrophilic layers are covered in unstable cationic liposomes, like in PEGylation, show reduced cellular interaction and cytotoxicity. Moreover, part-dehydrated and collapsed polymer chains produced liposomal aggregates, enhanced interactions between the liposomes and cell membranes, and rapidly released siRNA. In other words, near the LCST, the temperature-responsive polymer modification on cationic liposomes both stabilizes, like PEGylation below the LCST, and enhances higher cellular uptake and rapid release above the LCST. The changing polymer conformation rate by temperature might be a key factor to control cellular uptake.

Thus, modification with NIPAAm copolymers on conventional cationic liposome carriers for siRNA delivery might enhance gene-silencing activity. Moreover, when using temperature-responsive liposomes as carriers for gene transfection, the incubation temperature can be altered on demand since the polymer LCST can be designed. If gene transfection occurs at lower temperatures, cytotoxicity is reduced. This study provides future prospects that gene silencing potency, via NIPAAm copolymer-modified particles, can be controlled by temperature because tuning the surface properties from hydrated to dehydrated state of the complex may manipulate the endocytic pathways and the release siRNA into the cytosol.

4.4. Conclusion

I successfully developed temperature-responsive liposomes using [P(NIPAAm-co-DMAPAAm)] conjugated lipids as a carrier for siRNA delivery. The temperature-responsive liposomes enhanced gene silencing activity more than non-modified and PEGylated liposomes, comparable to the commercial transfection reagent, Lipofectamine RNAiMAX, with lower cytotoxicity at 37 °C. This high gene silencing activity by the temperature-responsive lipoplexes was observed at 37 °C, but not at 30 °C, and was related to cellular uptake, which was dependent largely on a microtubule-dependent transport, and also on the clathrin-mediated endocytosis pathway. This is the first report showing that temperature-responsive polymer-modified cationic liposomes thermally enhance cellular uptake and silencing activity of siRNA. The dehydrated polymer on the liposomes, and their aggregates caused by a decrease of the hydrated layers on the surface near the LCST may contribute to effective cellular uptake of the lipoplex for gene silencing activity by interaction with cell membrane.

Summary

In this study, I synthesized the temperature-responsive polymer and developed the temperature-responsive liposome as a novel carrier for siRNA delivery.

This temperature-responsive liposome had the LCST near body temperature. Changing of the surface of this temperature-responsive liposome from hydrophilic to hydrophobic and the liposomes showed the aqueous layer at the surface below the LCST, and FALT was decreased with temperature increasing.

The temperature-responsive liposomes showed significantly higher gene silencing activity than other carriers with less cytotoxicity. Furthermore, I showed that the temperature-responsive lipoplexes were internalized mainly via microtubule-dependent transport and also by the clathrin-mediated endocytosis pathway. This is the first report that temperature-responsive polymer-modified liposomes thermally enhanced silencing activity of siRNA.

Based on the results indicating that the surface of the liposomes was dehydration above LCST. The dehydrated polymer on the liposomes, and its aggregation can probably be attributed to effective cellular uptake of the lipoplexes for gene silencing activity by interaction with the cell membrane.

This is the first report showing that temperature-responsive polymer-modified cationic liposomes thermally enhance cellular uptake and silencing activity of siRNA. I hope that this study could improve the research of biological and medical field, and clinical application.

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Patent

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